AD_____

Award Number: DAMD17-00-2-0032

TITLE: Effect of Pretreatment With Human Butyrylcholinesterase Scavengers on the Toxicokinetics and Binding of Nerve Agents in Guinea Pigs and Marmosets

PRINCIPAL INVESTIGATOR: Jan P. Langenberg, Ph.D.
Hendrik P. Benschop, Ph.D.
Doctor M. J. van der Schans
Doctor L. P. A. de Jong

CONTRACTING ORGANIZATION: TNO Prins Maurits Laboratory
Rijswijk, 2280 AA, The Netherlands

REPORT DATE: June 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Annual (1 Jun 01 - 31 May 02)

4. TITLE AND SUBTITLE
Effect of Pretreatment With Human Butyrylcholinesterase Scavengers on the Toxicokinetics and Binding of Nerve Agents in Guinea Pigs and Marmosets

5. FUNDING NUMBERS
DAMD17-00-2-0032

6. AUTHOR(S)
Jan P. Langenberg, Ph.D.
Hendrik P. Benschop, Ph.D.
Doctor M. J. van der Schans
Doctor L. P. A. de Jong

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
TNO Prins Maurits Laboratory
Rijswijk, 2280 AA, The Netherlands

E-Mail: langenberg@pml.tno.nl

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)***

The effect of i.m. administered human butyrylcholinesterase (HuBuChE) on the i.v. bolus and inhalation toxicokinetics of (±)-sarin in guinea pigs was studied. After i.m. administration of HuBuChE, the BuChE activity in blood gradually increased, reaching a maximum after 20-24 h, and remaining stable for about another 24 h. It was decided to study the effect of HuBuChE on the toxicokinetics of nerve agent at 24 h after administration of the enzyme.

After iv. bolus administration of (±)-sarin, only the (-)-isomer was detectable in blood. The effect of HuBuChE-pretreatment (at a molar equivalent of 1.4 LD50 (i.v.) of C(±)P(±)-soman in the guinea pig) on the concentration-time profiles of (±)-sarin (2 LD50) was not as dramatic as anticipated. The most pronounced effect on the blood concentration of (-)-sarin in HuBuChE-pretreated guinea pigs was observed in the first 10 min after administration of the toxicant. The area-under-the-curve of (-)-sarin from time 0 to 20 min was significantly lower in HuBuChE-pretreated animals. Furthermore, via fluoride-induced reactivation a significantly higher concentration of (±)-sarin bound in blood was demonstrated in these animals.

After nose-only exposure to (±)-sarin vapor in air (*ca.* 2 LCt50 in 2 min) the effect of HuBuChE-pretreatment (at a molar equivalent of 0.5 LCt50 of C(±)P(±)-soman) on the toxicokinetics of (-)-sarin was also not as dramatic as anticipated.

In vitro, HuBuChE showed only minor stereoselectivity towards the nerve agents (±)-sarin, (±)-VX and C(±)P(±)-soman.

14. SUBJECT TERMS	15. NUMBER OF PAGES
human butyrylcholinesterase, scavengers, toxicokinetics, nerve agents, guinea pig	50
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

ACKNOWLEDGEMENTS

The authors wish to thank the people who are involved in this study: Dr. Hendrik P. Benschop, Dr. Daan Noort, Mrs. Dr. Carolien M. Boone, Mrs. J. Alice Cordia, Mrs. Corry van Dijk, Mrs. Herma J. van der Wiel , Mrs. Helma E.T. Spruit, Mr. Alex Fidder, Mr. Ton L. van der Laaken, Mr. Kees Pleijsier, and Mr. Henk C. Trap.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF TABLES

LIST OF TABLES (continued)

I. INTRODUCTION

An effective therapy against intoxication with nerve agents has been developed involving combined administration of atropine, oxime and anticonvulsant. Administration of the therapeutic agents almost immediately after intoxication is a prerequisite for their efficacy, which renders this approach less satisfactory under field conditions. The treatment is *a fortiori* much less effective if the nerve agent forms an inhibited acetylcholinesterase (AChE) that is, or rapidly becomes, resistant to reactivation by an oxime. Furthermore, even if the treatment would protect against the lethal effects of a nerve agent intoxication, the combined administration of atropine, oxime and anticonvulsant does not prevent the occurrence of post-exposure incapacitation.

Additional pretreatment with carbamates, such as pyridostigmine and physostigmine, has only partly solved these problems. The protection offered by carbamates is based on reversible inhibition of AChE, which at the same time shows the limits of pretreatment with inherently toxic compounds. The requirement for the application of a sign-free dose is contradictory to aiming at complete protection. Furthermore, the peripherally acting pyridostigmine, which is in use as the standard pretreatment drug, does not protect against post-exposure incapacitation. Application of the centrally acting physostigmine may prevent incapacitating effects of nerve agent intoxication but on the other hand will increase the possibility of adverse effects on the military performance of individuals not exposed to any nerve agent.

Therefore, attention has been paid to pretreatment with highly reactive scavengers, which would intercept or destroy the nerve agent before it could reach its target site, when entering the blood stream. It may be expected that effective scavengers offer protection against both lethal and incapacitating effects of an acutely toxic dose. In addition, if a scavenger remains in circulation at an effective concentration during a relatively long period of time, the pretreatment will, *a fortiori*, protect against a long term exposure to low doses of a nerve agent (Benschop *et al.*, 1998). It is further anticipated that bioscavengers do not induce adverse physiological effects, particularly when bioscavengers from human origin are applied.

As early as 1957, Cohen and Warringa achieved some protection in rats against a lethal subcutaneous dose of diisopropyl phosphorofluoridate and sarin by pretreatment with an enzyme capable of hydrolyzing organophosphates. In more recent years, the feasibility of using bioscavengers that can rapidly bind nerve agents has been studied, such as monoclonal antibodies (Lenz *et al.*, 1984; Brimfield *et al.*, 1985), fetal bovine serum AChE (Wolfe *et al.*, 1987; Ashani *et al.*, 1991; Maxwell *et al.*, 1992; Wolfe *et al.*, 1992) and human plasma butyrylcholinesterase (HuBuChE) (Ashani *et al.*, 1993; Raveh *et al.*, 1997; Allon *et al.*, 1998).

Very promising results were obtained with HuBuChE as a scavenger. The enzyme is rapidly distributed in laboratory animals, such as mice, rats, guinea pigs, and rhesus monkeys, after i.v. administration, followed by a slow elimination (Ashani *et al.*, 1993). In addition, the enzyme is sufficiently absorbed following an i.m. administration to provide therapeutically significant blood levels over 10 – 70 h in laboratory animals, which is a prerequisite for practical application. The peak level in blood after i.m. administration amounted to 50-60 % of the concentration obtained immediately after i.v. administration of the same amount of enzyme (Ashani *et al.*, 1993). Pretreatment with the enzyme resulted not only in an increase in survival of mice, rats and rhesus monkeys intoxicated (i.v.) with C(±)P(±)-soman or other nerve agents, but also in a significant alleviation of post-exposure incapacitation. An effective protection of guinea pigs against respiratory exposure to C(±)P(±)-soman has recently been reported (Allon *et al.*, 1998). Moreover, since the efficacy of HuBuChE as a scavenger is based on the inhibitory properties of the challenging agent, it can be expected that such scavengers will be effective against nerve agents having a wide variety of chemical structures.

The final aim is the use of this enzyme as a scavenger for protection of humans against acute intoxication by nerve agents. Although the results obtained in laboratory animals indicate the possible usefulness of HuBuChE as a scavenger, the information reported so far is insufficient for a thorough and quantitative description of the protective mechanism. Consequently, a reliable extrapolation of the working mechanism to human beings is not yet possible, although this will be needed for further development of the enzyme as a pretreatment drug for application in humans. Additional information is needed on at least three aspects that are elucidated in the following.

A. Toxicokinetics of nerve agents
The effect of nerve agent intoxication on the scavenger activity in blood of laboratory animals has been reported (Ashani *et al.*, 1993; Allon *et al.*, 1998). In addition to the decrease of HuBuChE activity in blood, the time course of the other reactant, i.e., the nerve agent, is essential in order to describe the interaction between the enzyme and the nerve agent adequately. These experiments will answer the key question with regard to the acceleration of nerve agent scavenging by HuBuChE down to levels that are toxicologically no longer relevant.

B. Stereoselectivity of HuBuChE inhibition
The common nerve agents are chiral organophosphates. The stereoisomers of these compounds may differ largely in their anti-butyrylcholinesterase activities. Therefore, rate constants for inhibition of HuBuChE by the single stereoisomers should be available. In contrast with AChE (Benschop and De Jong, 1988), these parameters are hardly known for inhibition of BuChE.

C. Binding in extravascular compartments
After i.m. administration, the enzyme will be distributed between the peripheral compartments and the central compartment prior to intoxication in the protection experiments. Furthermore, a fraction of the nerve agent may escape the scavenger circulating in the blood. Therefore, information on the fate of HuBuChE in extravascular compartments and of the relative importance of (additional) binding sites is needed for a thorough description of the protective mechanism of the scavenger.

The proposed studies should provide data that is lacking for the three above-mentioned items. Our proposed studies will be focussed on C(±)P(±)-soman, but some investigations on (±)-sarin and (±)-VX will also be performed.

Ad A. Toxicokinetic studies
During the last decade, we have performed various studies on the toxicokinetics of the stereoisomers of nerve agents in rat, guinea pig and marmoset (Benschop and De Jong, 1991; Benschop *et al.*, 1995, 1998; Due *et al.*, 1993, 1994; Langenberg *et al.*, 1998; Spruit *et al.*, 2000; Van der Schans *et al.*, 2000). For this purpose, we developed analytical procedures which allow the quantitation of nerve agent stereoisomers in blood and tissues at levels down to and below those of toxicologic relevance. An estimation of a toxicologically relevant level was based on the rationale (Benschop *et al.*, 1987) that only a small fraction (5-10 %) of active AChE has to be reactivated, e.g., by oximes, after total inhibition by a nerve agent in order to sustain vital functions. A nerve agent concentration that can reinhibit this fraction, will have a toxicologically relevant effect.

One of the conclusions from these studies is that from a toxicokinetic point of view the guinea pig is a better model for a primate than the rat. The studies performed in guinea pigs include determinations of time courses of the stereoisomers in blood after administration of supralethal i.v. bolus doses of C(±)P(±)-soman and after administration of sublethal doses of C(±)P(±)-soman and (±)-sarin both by an i.v. bolus injection and by respiratory exposure. Studies in which guinea pigs were exposed nose-only for a long period of time (300 min) to a low level of C(±)P(±)-soman (20 ppb) were also included. Short term and long term nose-only exposure to

C(±)P(±)-soman and (±)-sarin in guinea pigs are routinely performed in our laboratory (Benschop *et al.*, 1995; Benschop *et al.*, 1998; Langenberg *et al.*, 1998; Spruit *et al.*, 2000), whereas the same technique was applied to exposure to marmosets after small modification of the exposure chamber (Van Helden *et al.*, 2000). Studies on the toxicokinetics of the stereoisomers of (±)-VX after a lethal i.v. bolus in hairless guinea pigs and marmosets and a lethal percutaneous dose in hairless guinea pigs were recently concluded (Van der Schans *et al.*, 2000) within the context of Cooperative Agreement DAMD17-97-2-7001.

The results obtained from the abovementioned toxicokinetic studies were intended to serve as reference values for the newly proposed investigations on the efficacy of HuBuChE as a nerve agent scavenger.

We proposed to protect the animals by i.m. administration of HuBuChE, since this route is much more appropriate than i.v. administration for application of a scavenger in a realistic scenario. The ratio of the dose of HuBuChE relative to the dose of the nerve agent was chosen on the basis of results reported by Ashani *et al.* (1993) and Allon *et al.* (1998) in order to obtain sufficient protection in a similar experiment. The challenge dose of the nerve agent was chosen to be twice a lethal dose, in accordance with the experimental setup of most of the experiments reported by Ashani and coworkers. Moreover, in recent experiments on therapeutic efficacy of oximes (Pearce *et al.*, 1999), protection against twice the lethal dose has been accepted as a realistic goal.

Three series of toxicokinetic studies are performed. In the first series of experiments, the toxicokinetics of the stereoisomers of C(±)P(±)-soman, (±)-sarin and (±)-VX are studied following an i.v. bolus administration of the agent in guinea pigs pretreated with HuBuChE. In these experiments the total amount of nerve agent entering the animals will be known accurately, which is important for a reliable description of the protective mechanism of the scavenger. In addition, toxicokinetics will be studied after a repeated i.v. dose of C(±)P(±)-soman (2 LD50) in order to expand the dose range for which to obtain data on the protective activity of the scavenger.

In the second series of toxicokinetic studies guinea pigs are exposed to the nerve agents by the most probable way of intoxication when used under realistic conditions, i.e., exposure via inhalation for the more volatile agents C(±)P(±)-soman and (±)-sarin and via the skin for the nerve agent (±)-VX. The toxicokinetics of the stereoisomers of C(±)P(±)-soman and (±)-sarin will be studied following short term (2 min) nose-only exposure of pretreated guinea pigs to these two agents at doses corresponding with 2 LCt50. In order to investigate the efficacy of HuBuChE scavenger in long term-low level exposure to nerve agents, the time course of the stereoisomers of C(±)P(±)-soman will also be determined during and after a 300-min exposure of HuBuChE-pretreated guinea pigs to 2 LCt50 of C(±)P(±)-soman. Furthermore, the toxicokinetics of the stereoisomers of (±)-VX will be studied after percutaneous administration of 2 LD50 of the agent to HuBuChE-pretreated hairless guinea pigs.

Extrapolation to humans is more reliable if toxicokinetic data obtained in non-human primates are available. Therefore, a third, but limited, study will be performed on the toxicokinetics of the stereoisomers of C(±)P(±)-soman following respiratory exposure of HuBuChE-pretreated marmosets to the agent. As a reference, the toxicokinetics will be studied in non-protected animals challenged with 0.8 LCt50 of C(±)P(±)-soman. The LCt50 value for C(±)P(±)-soman in marmosets will be calculated as the estimated LCt50 values for C(±)P(±)-soman in humans (Black and Harrison, 1996) corrected for marmosets by multiplying with the ratio between the value for (±)-sarin in monkeys (Marrs *et al.*, 1996) and in humans (Black and Harrison, 1996).

Ad B. Stereoselectivity of HuBuChE inhibition
Rate constants for inhibition of HuBuChE by the stereoisomers of C(±)P(±)-soman are determined by using the single stereoisomers at first-order or second-order conditions, according to common procedures. The stereoisomers are isolated according to procedures well-known in our laboratory (Benschop *et al.*, 1984). The inhibition reactions with (±)-sarin and (±)-VX will be performed by using the racemic mixtures of the nerve agents. Kinetic analysis of the inhibition of HuBuChE performed at second-order conditions will allow to derive the rate constants for the individual stereoisomers (Boter and Van Dijk, 1969).

Ad C. Binding in extravascular compartments
Indications for binding of the nerve agent to extravascular HuBuChE in pretreated guinea pigs intoxicated by C(±)P(±)-soman were obtained from preliminary studies using a physiologically based model which describes the toxicokinetics of C(±)P(±)-soman after i.v. administration in guinea pigs. This model has been developed as a cooperative effort of TNO Prins Maurits Laboratory and U.S. Army Medical Research Institute of Chemical Defense (Langenberg *et al.*, 1997). Recently, we performed preliminary calculations on the influence of HuBuChE scavenger on the toxicokinetics of C(±)P(±)-soman and on the inhibition of the scavenger (De Jong *et al.*, 2000). The rate constants for reaction of the C(±)P(±)-soman stereoisomers with the scavenger were estimated from the overall rate constant for inhibition of HuBuChE by C(±)P(±)-soman (Ashani *et al.*, 1993) and from the relative potencies of the stereoisomers as determined for horse serum BuChE (Keijer and Wolring, 1969). The dose of i.v. administered C(±)P(±)-soman corresponded with 2 LD50, whereas the dose of scavenger was taken from Ashani's study (1993) yielding complete protection. Assuming that the scavenger circulated *only* in the central compartment, the model predicts that the residual enzyme activity is very low (< 5%), whereas the observed HuBuChE activity in rats and rhesus monkeys is relatively high (40-45 %).

We decided to perform the following three series of experiments in order to obtain information on the effect of nerve agent administration on the fate of HuBuChE in blood and in extravascular compartments of the guinea pig.
In the first series, the distribution of HuBuChE over blood, lung, liver, kidney and brain is determined at the point of time after i.m. administration to guinea pigs at which the nerve agent is administered in the toxicokinetic experiments. HuBuChE concentrations in blood and in homogenates of the selected tissues will be determined on the basis of enzyme activity.
In the second series, samples of the same tissues of guinea pigs pretreated with the enzyme (i.m.) are taken 1 min after i.v. administration of a dose corresponding with 2 LD50 of C(±)P(±)-^{14}C-soman (labeled in the phosphonomethyl group). It will be attempted to separate inhibited (and noninhibited) HuBuChE from other binding sites to which C(±)P(±)-^{14}C-soman has been bound in tissues and in blood by means of affinity chromatography on procainamide sepharose (Ashani *et al.*, 1993). Preliminary experiments in our laboratory have shown that elution of C(±)P(±)-soman-inhibited HuBuChE from the gel is retarded in a similar way as elution of the noninhibited enzyme. The collected fraction will be tested for radioactivity and HuBuChE activity.
The third series comprises similar experiments, but the determinations will be carried out in samples taken 90 min after C(±)P(±)-^{14}C-soman administration.

Modeling studies
Finally, information obtained on the rate constants for inhibition of HuBuChE by the stereoisomers of C(±)P(±)-soman and on the distribution of the enzyme over various organs will be introduced into the physiologically based model for the i.v. toxicokinetics of C(±)P(±)-soman in the guinea pig (Langenberg *et al.*, 1997; De Jong *et al.*, 2000). The model will be validated by comparing simulated levels of the toxic C(±)P(-)-soman isomers in blood and of C(±)P(±)-soman bound to HuBuChE after an i.v. bolus administration of the nerve agent with the levels determined in the proposed experiments.

II. EXPERIMENTAL PROCEDURES

II.1 Materials

(±)-Sarin (isopropyl methylphosphonofluoridate), C(±)P(±)-soman (1,2,2-trimethylpropyl methylphosphonofluoridate), C(+)P(±)-soman, C(-)P(±)-soman, (±)-VX (O-ethyl S-(2-diisopropylaminoethyl) methylphosphonothioate), and D7-(±)-sarin were obtained from the stocks at TNO Prins Maurits Laboratory.
Human Butyrylcholinesterase was obtained from Dr. Doctor from Walter Reed Army Institute of Research (Washington DC). The enzyme, dissolved in glycerol, was transferred into phosphate buffered saline (PBS) via dialysis (4 °C, overnight), after which the volume was reduced by leading the solution through a 30 kD cut-off filter (Schleicher & Schuell, Keene, NH, USA).
Dormicum® was purchased from Roche (Mijdrecht, The Netherlands), Hypnorm® from Solvay-Duphar (Amsterdam, The Netherlands), Vetranquil® and Nembutal® from Sanofi (Maassluis, The Netherlands), and heparine, veronal sodium and atropine sulfate from Brocacef (Rijswijk, The Netherlands).
Analytical grade ethyl acetate, 2-propanol, acetone, acetic acid, sodium acetate, sodium chloride, sodium hydroxide, disodium hydrogen phosphate, potassium dihydrogen phosphate, aluminium sulfate, potassium fluoride, sodium dodecylsulfate, α-chymotrypsin and saponin were obtained from Merck (Darmstadt, Germany). Butyrylthiocholine iodide and dithionitrobenzoic acid were purchased from Aldrich (Brussels, Belgium).
Rabbit serum was procured from Harlan NL (Horst, The Netherlands).
Nexus® solid phase extraction cartridges were purchased from Varian (Middelburg, The Netherlands). Extrelut 20 was procured from Merck.

II.2 Methods

II.2.1 Activity of butyrylcholinesterase in blood

Blood samples were diluted tenfold with a solution of 1% saponin in 100 mM phosphate buffer (pH 7.5). Subsequently, the diluted samples were frozen in liquid nitrogen if they were not analyzed immediately.
BuChE activity was measured according to the colorimetric method described by Ellman *et al.* (1961), using butyrylthiocholine iodide as a substrate.

II.2.2. Sample preparation of (±)- sarin from blood

Blood samples (1 part) were mixed with three parts of stabilization buffer (0.2 M sodium acetate, pH 3.5, 1.6 mM aluminum sulfate, 1 µg C(±)P(±)-soman/ml), after which the internal standard (D7-sarin in 2-propanol) was added. The mixture was transferred onto a Nexus® solid phase extraction cartridge (200 mg) and eluted with 2 ml ethyl acetate. The ethyl acetate layer was separated from the aqueous layer by freezing the aqueous layer with a dry ice/acetone mixture. The ethyl acetate phases were transferred onto Tenax for analysis with the GLC-configuration described in paragraph II.2.4.

II.2.3 Fluoride-induced reactivation of sarin bound to esterases in blood

The phosphyl moiety of sarin as bound to esterases and probably other binding sites in blood was reactivated with fluoride ions according to the procedure as described by Polhuijs *et al.* (1997). The procedure was slightly modified, i.e., the Sep-Pak C18 cartridge was replaced with a 200-mg Nexus® SPE-cartridge, which provided for a higher and more reproducible recovery of (±)-sarin. The ethyl acetate eluates from the Nexus® cartridges were transferred onto Tenax for analysis with the GLC-configuration decribed in paragraph II.2.4.

II.2.4. Gas chromatographic analysis of (±)-sarin

The gas chromatographic system used to analyze the stereoisomers of (±)-sarin consisted of a Carlo Erba HRGC 5160 Mega Series (Milan, Italy), equipped with an alkali flame detector (NPD, Carlo Erba), a thermodesorption cold trap injector (TCT, Chrompack, Middelburg, The

Netherlands), and a Chrompack MUSIC system (Multiple Switchable Intelligent Controller) for two-dimensional gas chromatography.
The desorption tubes (length 15 cm, i.d. 2.5 mm) were partly (ca. 80 %) filled with Tenax TA 60-80 mesh (Varian). A glass wool plug was firmly pushed on top of the Tenax material and was fixed with a metal clamp. The tubes were preconditioned by heating under a stream of nitrogen at 300 °C for at least 4 h.
The pre-column of this system was CPSil 8 CB, (length 30 m i.d. 0.53 mm, film thickness 5 µm). The analytical column was a CP-Cyclodextrin-B2,3,6-M-19 column (Chrompack, length 50 m, 0.25 mm i.d., film thickness 0.25 µm).
Tenax tubes were loaded with sarin samples in ethyl acetate (400 µl) in portions of 100 µl. In between each loading the tube was flushed with nitrogen (240 ml/min) for three min. Next, the tube was placed in the thermodesorption device and sarin was desorbed by heating the tube for 6 min at 200 °C. Sarin was focussed in a cold trap which was cooled down to –60 °C with liquid nitrogen. The cold trap consisted of 1 m CPSil 8 CB (i.d. 0.53 mm). Flow (helium) through the pre-column was 16 ml/min. Cooling was started before the tube was placed. Sarin was injected onto the pre-column by flash heating from –60 °C to 220 °C at a rate of 21 °C/sec; the temperature of the TCT-injector was maintained for 10 min. The effluent of the pre-column was monitored with an FID detector. The effluent of the pre-column within the time interval of 7.1 and 9.7 min was focussed in a second cold trap, which was cooled with liquid carbon dioxide, controlled by the MUSIC system. Subsequently, sarin was injected onto the analytical column. The flow through the analytical column was pressure-controlled and was kept at 106 kPa.
Overall oven temperature program of the GC: Initial temperature 70 °C maintained for 6 min, raised to 90 °C at a rate of 10 °C/min, cooled down to 70 °C at infinite rate, maintained at 70 °C for 5 min and raised to 82 °C at a rate of 0.5 °C/min. Next, the temperature was raised to 200 °C and maintained for 5 min. The detector base temperature was kept at 250 °C. Make-up gas for the NP-detector was helium at a flow-rate of 38 ml/min. Flow-rates of air and hydrogen through the NP-detector were 350 and 35 ml/min, respectively.

II.2.5 Isolation of individual nerve agent stereoisomers

II.2.5.1 Isolation of (-)-D7-sarin from (±)-D7-sarin
100 µl of (±)-D7-sarin solution in 2-propanol was incubated in 12 ml rabbit serum for 30 s at 37 °C. Next the mixture was poured onto a bed of 7.5 g Extrelut 20. After absorbing the fluid, the bed was rinsed with 35 ml of ethyl acetate. Ethyl acetate containing (-)-D7-sarin was recovered. Its concetration was determined with the GLC-configuration described in II.2.4.

I.2.5.2. Isolation of soman isomers
Soman isomers were isolated analogously to methods described by Benschop *et al.* (1984). GC anaysis on Chirasil-Val was used to quantify the concentrations of the isomers in the final solution and to check their optical purities.

- Isolation of C(-)P(-)-soman from C(-)P(±)-soman

C(-)P(±)-soman (10 mg) was incubated in rabbit serum (25 ml) for 50 s at 37 °C. Next, the serum was poured onto a bed of 25 g Extrelut 20. After the fluid being absorbed by the Extrelut, the soman isomer was extracted with 70 ml of ethyl acetate. The recovered ethyl acetate was evaporated under reduced pressure (44-50 mm Hg) to a residual volume of approx. 50 µl and diluted to a final volume of 1 ml with acetonitrile. Chiral GLC-analysis revealed that this preparation of
C(-)P(-)-soman contained *ca.* 2% of the C(+)P(-)-soman isomer.

- Isolation of C(-)P(+)-soman from C(-)P(±)-soman

C(-)P(±)-soman (10 mg) was incubated in 35 ml phosphate buffer (0.01M, pH 6.2) containing 1.75 g α-chymotrypsin. After 60 s of incubation the mixture was poured onto a bed of 30 g Extrelut 20. After equilibration, the bed was rinsed with 110 ml ethyl acetate. The recovered

ethyl acetate was evaporated in a rotary evaporator under reduced pressure (45-50 mm Hg) to a residual volume of approx. 100 µl. Next, the solution was diluted to 1 ml with acetonitrile. Chiral GLC-analysis revealed an optical purity of this C(-)P(+)-soman preparation of 99.9%.

- Isolation of C(+)P(-)-soman from C(+)P(±)-soman

C(+)P(±)-soman (10 mg) was incubated for 25 s in 12.5 ml of rabbit serum at 37 °C. The mixture was poured onto a bed of 7.5 g Extrelut 20, After equilibration the bed was rinsed with 37.5 ml ethyl acetate. The recovered ethyl acetate was evaporated in a rotary evaporator under reduced pressure (45-50 mm Hg) to a residual volume of *ca.* 100 µl. Next, the solution was diluted to a final volume of 1 ml with acetonitrile. Chiral GLC-analysis revealed an optical purity of this C(+)P(-)-soman preparation of 99.7%.

- Isolation of C(+)P(+)-soman from C(+)P(±)-soman

C(+)P(±)-soman (10 mg) was incubated in 40 ml phosphate buffer (0.01M, pH 6.2) containing 3.48 g α-chymotrypsin. After 60 s the mixture was poured onto a bed of 40 g Extrelut 20. After absorption of the fluid, the bed was rinsed with 110 ml of ethyl acetate. The recovered ethyl acetate was evaporated in a rotary evaporator under reduced pressure(45-50 mm Hg) to a residual volume of *ca.* 100 µl. Next, the solution was diluted to 1 ml with acetonitrile. Chiral GLC-analysis revealed an optical purity of this C(+)P(+)-soman preparation of 99.9%.

II.2.6 Determination of rate constant of inhibition of HuBuChE by the stereoisomers of the nerve agents(±)- VX, (±)-sarin and C(±)P(±)-soman

HuBuChE was dissolved in 50 mM veronal buffer, pH 7.4, containing 0.1 M sodium chloride, to the desired concentration. Inhibition was started by addition of a small volume (sarin, VX) or an equal volume (soman isomers) of a solution of the organophosphate at the desired concentration in the same buffer. Aliquots (30-60 µl) were taken at various times of incubation at 38 °C and added to a solution (4 ml) of 0.4 mM butyrylthiocholine iodide and 0.4 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 0.05 M phosphate buffer, pH 8.0. After reacting at 25 °C for 10 min [(±)-sarin, (±)-VX] or 20 min (C(±)P(±)-soman stereoisomers), the substrate hydrolysis was stopped by addition of a solution of 13% sodium dodecylsulfate in water (0.15-0.20 ml). Subsequently, the extinction of the solution was measured at 412 nm after approximately 3 min.

II.2.7 Animals

Male albino outbred guinea pigs of the Dunkin-Hartley type (species identification: Crl:(HA)BR), weighing 350-400 g were purchased from Harlan NL (Horst, The Netherlands). Health certificates were examined before delivery was approved and were subsequently archived. The animals were housed in temperature- and humidity-controlled rooms. They were allowed to eat and drink *ad libitum.* Teklad® guinea pig food was procured from Harlan NL. Analysis reports of the food batches were received, inspected and filed.
Two standard operation procedures were applicable to the care of the guinea pigs, i.e., 'Ordering and Housing of Experimental Animals' (SOP Q213-W-039) and 'Cleaning and Maintenance of Animal Facilities' (SOP Q213-W-040).
The animals were allowed to acclimatize to their new environment for at least 1 week before they were used in any experiment. Mostly, the guinea pigs were in the weight range of 500-700 g when they were used in the experiments.
The protocol for the animal experiments was approved in August 2000 by the TNO Animal Experiment Committee under number DEC 834.

II.2.8 Administration of HuBuChE to the guinea pig

24 h before the start of a toxicokinetic experiment, guinea pigs were injected i.m. in the hind legs with a dose of HuBuChE dissolved in phosphate buffered saline (PBS). The maximum volume injected was 600 µl, divided over both hind legs. The dose of HuBuChE depended on the type of experiment (i.v. bolus or nose-only exposure) and the weight of the animal.

In dose-finding experiments, guinea pigs were anesthetized and a carotid cannula was inserted which protruded from the neck. Heparine (5000 U) was administered via the cannula. Blood samples were drawn via the cannula just prior to administration of HuBuChE and at various time points after i.m. administration of this scavenger. At the end of the experiment the animals were euthanized with an overdose of Nembutal® (0.4 ml, i.p.)

II.2.9 Toxicokinetics of (±)-sarin in the anesthetized, atropinized and artificially ventilated guinea pig following intravenous bolus administration

Guinea pigs (pretreated with HuBuChE) were anesthetized with a mixture (3.5 ml/kg, i.m.) of Dormicum® (midazolam 5 mg/ml) and Hypnorm® (fentanyl 0.315 mg/ml and fluanisone 10 mg/ml). Additionally, 0.2 ml of Vetranquil® (acepromazine maleate 10 mg/ml) was injected i.m. Cannulas were inserted into the left carotid artery and into the trachea. Heparin (5000 U) was administered via the carotid cannula. The animals were mechanically ventilated using an infant ventilator (Hoek Loos, Schiedam, The Netherlands).

The jugular vein was traced and made accessible. Atropine sulfate, dissolved in sterile saline, was administered i.p. (17.4 mg/kg). A blood sample was drawn via the carotid cannula, after which a volume of sterile saline corresponding with that of the blood sample was administered through the same cannula. A dose of (±)-sarin corresponding with 2 LD50 (48 µg/kg) was injected into the jugular vein (injection volume 1 ml/kg). For this purpose a standard solution of sarin in 2-propanol was diluted with sterile saline just before use. Blood samples were drawn at various time points up to 120 min after administration of the toxicant. A corresponding volume of saline was given back after each sampling event. After taking the final blood sample the animals were sacrificed with an overdose of pentobarbital sodium (Nembutal®, 0.4 ml, i.p).

A small portion of the blood sample was used to determine the BuChE activity, whereas the larger part was used for gas chromatographic analysis. On a portion of the final blood sample, the fluoride-induced reactivation procedure was applied, in order to quantify the total amount of (±)-sarin (free and bound) in the sample.

Since a reference toxicokinetic curve for 2 LD50 as an intravenous bolus was not yet available, similar experiments were performed with animals that were not pretreated with HuBuChE.

II.2.10 Toxicokinetics of (±)-sarin in the anesthetized and atropinized guinea pig during and after nose-only exposure to (±)-sarin vapor in air

Toxicokinetic experiments were performed by nose-only exposure of anesthetized, atropinized and restrained guinea pigs to a concentration of (±)-sarin vapor in air, yielding approximately 2 LCt50 in 2 min. The apparatus was basically the same as developed within the context of Cooperative Agreement DAMD17-90-Z-0034 (Benschop and Van Helden. 1993; Langenberg *et al.* 1998b). During the performance of Cooperative Agreement DAMD17-94-V-4009 (Langenberg et al. 1998a) the exposure module was modified in the sense that the internal volume was reduced and the pathways shortened, whereas the teflon front chamber from which the animal breathes was replaced with a stainless steel front chamber.

Guinea pigs (pretreated with HuBuChE) were anesthetized with a mixture (3.5 ml/kg, i.m.) of Dormicum® (midazolam 5 mg/ml) and Hypnorm® (fentanyl 0.315 mg/ml and fluanisone 10 mg/ml). Additionally, 0.2 ml of Vetranquil® (acepromazine maleate 10 mg/ml) was injected i.m. A cannula was inserted into the left carotid artery. Heparin (5000 U) was administered via the carotid cannula. Next, the animals were atropinized by i.p. administration of 1 ml/kg of a solution of 17.4 mg atropine sulfate per ml of saline. The animals were restrained in a modified Battelle tube (Langenberg *et al.* 1998b), with the carotid artery cannula protruding from the tube. Blood samples were taken just before starting the 2-min exposure and at various time points up to 60 min thereafter. After taking the final blood sample the animals were sacrificed with an overdose of pentobarbital sodium (Nembutal®, 0.4 ml, i.p).

A small portion of the blood sample was used to determine the BuChE activity, whereas the larger part was used for gas chromatographic analysis. On a portion of the final blood sample, the fluoride-induced reactivation procedure was applied, in order to quantify the total amount of (±)-sarin (free and bound) in the sample.

Since a reference toxicokinetic curve for approximately 2 LCt50 in a 2-min exposure was not available, similar experiments were performed with animals that were not pretreated with HuBuChE.

II.2.11 Curve-fitting of toxicokinetic data

Curve-fitting of the measured concentration-time courses was performed by nonlinear regression with TableCurve software (Jandell, AISN Software) on a personal computer equipped with an Intel Celeron 300 mHz processor, as decribed previously (Benschop and De Jong, 1990).
The data were fitted to a multiexponential equation :

$$[\text{nerve agent}]_t = A*e^{-at} + B*e^{-bt} + C*e^{-ct} \qquad \text{(eq. 1)}$$

by calculation of the parameters A, B, C, a, b, and c. In these equations, $[\text{nerve agent}]_t$ is the blood concentration of the nerve agent stereoisomer under study at time t.
Several toxicokinetic parameters were calculated:

area under the curve (AUC)	$AUC = A/\alpha + B/\beta$	(eq. 2)
total body clearance (Cl)	$Cl = Dose/AUC$	(eq. 3)
blood concentration at time 0(C_0)	$C_0 = A + B + C$	(eq. 4)
half-life of first toxicokinetic phase ($t_{½,a}$)	$t_{½,a} = \ln 2/a$	(eq. 5)
half-life of second toxicokinetic phase ($t_{½,b}$)	$t_{½,b} = \ln 2/b$	(eq. 6)
half-life of third toxicokinetic phase ($t_{½,c}$)	$t_{½,c} = \ln 2/c$	(eq. 7)
volume of the central compartment (V_1)	$V_1 = Dose/C_0$	(eq. 8)

III. RESULTS

III.1. TIME COURSE OF HuBuChE IN BLOOD OF THE GUINEA PIG FOLLOWING INTRAMUSCULAR ADMINISTRATION (T.O. 1)

In order to establish which point in time after administration of HuBuChE to guinea pigs would be optimal for a nerve agent challenge, the time course of BuChE activity was determined in blood samples drawn from three guinea pigs at various time points after administration of the enzyme, using the colorimetric method of Ellman *et al.* (1961). The results are shown in Table 1 and Figure 1.

Table 1. Activity of BuChE in blood of guinea pigs at various time points after intramuscular administration of HuBuChE.

Animal	Guinea pig 1		Guinea pig 2		Guinea pig 3	
Weight (g)	448		454		532	
HuBuChE dose (U/kg)		20700		20700		10500
	Time (h)	BuChE (U/ml)	Time (h)	BuChE (U/ml)	Time (h)	BuChE (U/ml)
	0	1.84	0	1.14	0	1.75
	1	1.92	1	2.20	1	4.54
	2	2.91	2	1.67	2	4.88
	18	30.8	18	33.3	19	21.9
	20	34.1	20	35.8	22	21.8
	22	36.3	22	34.0	26	22.9
	24	34.8	24	33.4	43	20.2
	26	38.1	26	34.5	46	21.8
	43	48.3	44.5	35.4	48	22.0
	46	53.7	49	29.6	50	21.6
	48	51.5	67	28.3	67	14.7
			73	26.0	71	15.6
					74	15.6

Note: 60 U corresponds with approximately 1 nmol

As expected, the baseline activities of BuChE in the blood of the three guinea pigs shows some variation. After i.m. administration of HuBuChE the BuChE activity in blood increases with a half-life of approx. 5 h (which largely corresponds with the half-life of absorption) up to 20-24 h after administration, after which the activity remains relatively constant for another 24 h. Subsequently, the activity declines with a half-life of *ca.* 60 h (which is largely the elimination half-life). In the studied dose range, the maximum BuChE activity that is reached appears to be linear with the dose.

We wish to emphasize here that we were not able to obtain adequate fits to the pharmacokinetic equation: $BuChE\ activity = A*exp(-k_{el}*t)-B*exp(-k_{abs}*t)+C$, in which k_{abs} is the absorption rate constant, k_{el} the elimination rate constant, and C the BuChE activity at time zero. This is mainly due to an insufficient number of data in the elimination phase of the curve.

In guinea pig #1 the pattern seems to be aberrant, as the BuChE activity increases considerably after levelling off around 20-24 h, for which we have no explanation at this point. It looks like this guinea pig received a second dose of HuBuChE, which however was not the case.

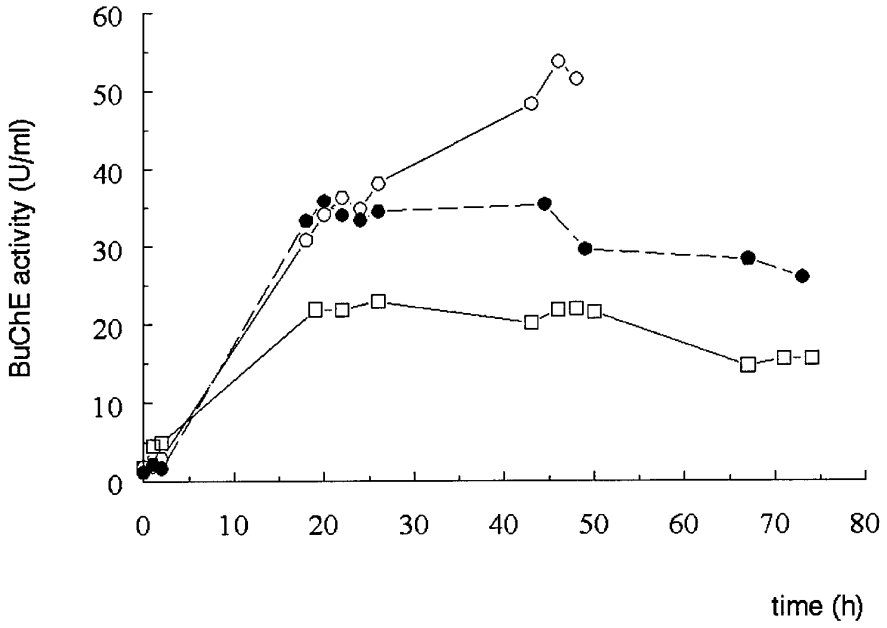

Figure 1. Concentration-time courses of BuChE-activity measured in blood of guinea pigs # 1 (○), 2 (●) and 3 (□), following intramuscular administration of HuBuChE.

III.2 TOXICOKINETICS OF (±)-SARIN STEREOISOMERS IN ANESTHETIZED, ATROPINIZED AND ARTIFICIALLY VENTILATED GUINEA PIGS PRETREATED WITH HuBuChE AFTER INTRAVENOUS BOLUS ADMINISTRATION (T.O. 4)

III.2.1. Gas chromatographic analysis of (±)-sarin stereoisomers

Initially the two-dimensional gas chromatography system was configured with a flow-controlled TCT injection system. Recently a new, pressure-regulated, TCT-injector became available, which we intended to use in this study. Unfortunately, we did not succeed in getting a reproducible injection with this set-up, most likely due to interference of the pressure-controlled TCT injection and the back-pressure from the MUSIC-system. Consequently, we reverted to the old, proven, configuration for trace analysis of sarin stereoisomers.
Furthermore, we encountered unexpected difficulties in finding Cyclodex columns that provided sufficient resolution between the sarin stereoisomers. According to the manufacturer all columns have the same specifications as verified with a test mixture. Apparently, the chiral resolution of (±)-sarin is more critical. We observed that around 70 °C the cyclodextrin phase underwent a phase transfer between solid and liquid. In order to obtain resolution between sarin stereoisomers, a liquid phase is needed. However, by increasing the temperature in order to ensure the cyclodextrin phase to be liquid, we had insufficient chromatographic resolution. The manufacturer gave us the opportunity to test a number of columns with (±)-sarin on our configuration, after which we were able to select a few specimens that met with our criteria. Detection limit of sarin was 5 pg per isomer (S/N=3). Based on the injection volume of 400 µl, concentrations down to 40 pg/ml could be determined with statistical significance (S/N=10). Analysis of blank blood samples showed no peaks with the same retention times as (+)-sarin, (-)-sarin or (-)-D7-sarin.

III.2.2 Toxicokinetics of (±)-sarin in blood of the guinea pig after intravenous administration of a dose corresponding with 2 LD50

Thusfar, the intravenous toxicokinetics of (±)-sarin have not been studied as extensively as those of C(±)P(±)-soman. In fact, only a dose corresponding with 0.8 LD50 (19.2 µg/kg) has been studied (Benschop and Van Helden, 1993; Spruit *et al.*, 2000). When we proposed this study it was our intention to compare the toxicokinetics for 2 LD50 in HuBuChE-pretreated guinea pigs with those for 0.8 LD50 in non-pretreated animals, assuming a dramatic effect on the toxicokinetics of HuBuChE pretreatment. As will become apparent, the effect of the scavenger on the toxicokinetics is not that dramatic. As a consequence we decided to perform a limited study on the toxicokinetics of 2 LD50 sarin in naive animals.
Sarin stereoisomers were measured in the blood of anesthetized, atropinized and artificially ventilated guinea pig after i.v. administration of a dose of (±)-sarin corresponding with 2 LD50, i.e. 48 µg/kg. Blood samples were drawn at time 0 (just before administration of (±)-sarin) and at 1, 2, 4, 10, 20, 40, 60 and 120 min after the bolus injection. The drawn blood volumes ranged from 0.3 up to 2 ml (for the final sample).
(+)-Sarin was not detected in any of the blood samples (< 40 pg/ml blood). The measured concentrations of (-)-sarin in the naive animals are listed in Table 2. The mean concentration-time course is shown in Figure 2.
Up to 20 min after the i.v. bolus injection the concentration of (-)-sarin in blood decreases as expected. Oddly enough, after that the concentration increases again, which means that under these conditions (-)-sarin is far more persistent *in vivo* than anticipated.

Table 2. Concentration (ng/ml) of (-)-sarin in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a dose of (±)-sarin corresponding with 2 LD50 (48 µg/kg).

Animal #	GB 1	GB 2	GB 3	GB 4	Mean ± SEM
Weight (g)	615	685	574	641	
Time (min)	[(-)-sarin]	[(-)-sarin]	[(-)-sarin]	[(-)-sarin]	[(-)-sarin]
1	8.18	4.24	24.0	6.96	10.8 ± 4.4
2	3.64	2.40	8.16	2.86	4.27 ± 1.3
4	1.00	1.00	1.69	0.77	1.12 ± 0.19
10	0.20	0.15	0.09	0.15	0.15 ± 0.02
20	0.10	0.11	0.20	0.13	0.13 ± 0.02
40	0.22	0.27	0.35	0.27	0.28 ± 0.03
60	0.20	0.25	0.61	0.30	0.34 ± 0.09
120	0.32	0.22	0.94	0.27	0.44 ± 0.17

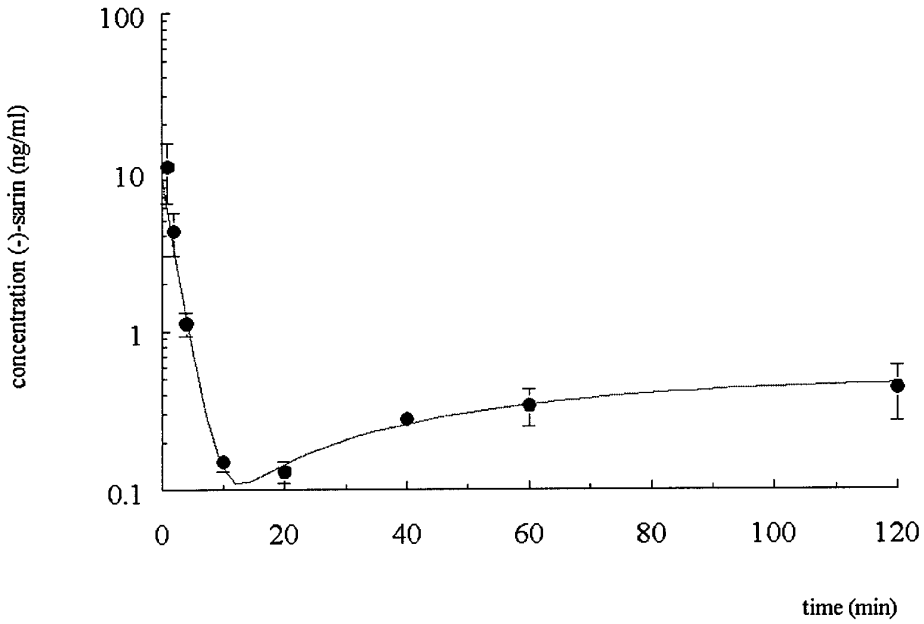

Figure 2. Mean concentration-time course of (-)-sarin (ng/ml ± SEM, n=4) in blood of anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. administration of a (±)-sarin dose of 48 µg/kg, which corresponds with 2 LD50.

The data in Table 2 were fitted to a three-exponential equation: $A.e^{-at}+B.e^{-bt}+Ce^{-ct}$. The rather peculiar looking concentration-time course can be fitted remarkably well to this equation as shown in Figure 2, albeit that one of the coefficients has a negative value. The toxicokinetic parameters derived from this fit are presented in Table 3.

Table 3 Toxicokinetic parameters for (-)-sarin in anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. bolus administration of a (±)-sarin dose corresponding with 2 LD50 (48 µg/kg).

Parameter	Dimension	Value
Dose	$ng.kg^{-1}$	48000
Number of exponents		3
A	$ng.ml^{-1}$	9.27
B	$ng.ml^{-1}$	-2.62
C	$ng.ml^{-1}$	2.61
a	min^{-1}	0.504
b	min^{-1}	0.00831
c	min^{-1}	0.00497
AUC(0-∞)[a]	$ng.min.ml^{-1}$	228
AUC(0-120')[b]	$ng.min.ml^{-1}$	55.5
AUC(0-20')[b]	$ng.min.ml^{-1}$	19.9
C0	$ng.ml^{-1}$	9.26
t½,a	min	1.37
t½,b	min	83.4
t½,c	min	140
Vl	$l.kg^{-1}$	5.2
Cl	$l.min.kg^{-1}$	0.864

[a] This value for the AUC was calculated from the coefficients and exponents of the curve-fit (paragraph II.2.11, equation 2)
[b] These values for the AUC were derived by numerical integration.

III.2.3 Toxicokinetics of (±)-sarin in blood of the HuBuChE-pretreated guinea pig after intravenous administration of a dose corresponding with 2 LD50

In a subsequent series of experiments, guinea pigs were pretreated with HuBuChE. At 24 h before administration of the toxicant, guinea pigs were injected with a dose of HuBuChE corresponding with 0.7 times the molar dose of 2 LD50 C(±)P(±)-soman (i.v.). The i.v. LD50 of C(±)P(±)-soman is 27.5 µg/kg (Benschop and De Jong, 1991), which corresponds with 0.15 µmol/kg. The dose of HuBuChE required for pretreatment therefore is 2*0.7*0.15=0.21 µmol HuBuChE/kg. Table 4 shows the amounts of HuBuChE that were actually injected into the individual animals, which deviated from the required amounts to some extent. The reason for this is that the recovery of HuBuChE from the glycerol preparation was not particularly constant in the early days of the study.
Furthermore, in Table 4 the concentrations of (-)-sarin measured in the various blood samples are presented, as well as in Figures 3 and 4. The (+)-isomer could not be detected in any of the blood samples.
Upon comparison of the data in Tables 2 and 4 it appears that generally the concentrations of (-)-sarin are lower in the HuBuChE-pretreated animals than in the naive animals. The difference is most prominent in the first few minutes after administration of (±)-sarin, but also in the terminal phase of the curves.
In experiments GBH 3 and 5, and presumably 2, (-)-sarin appears to be captured by the scavenger almost immediately. This is what we anticipated for the toxicokinetic curves to look like in the presence of HuBuChE.
Furthermore it is obvious from the BuChE data in Table 4, that there is quite a bit of active BuChE left at the end of the toxicokinetic experiment, whilst (-)-sarin is still circulating at low (but still easily detectable) concentrations.
Unfortunately, the variation in the results for (-)-sarin is such that the individual values should not be averaged in order to obtain a mean concentration-time course. Instead, each individual curve needs to be analyzed to obtain toxicokinetic parameters, which may then be averaged. However, we were not very successful in deriving meaningful toxicokinetic parameters from the results of these experiments (cf. footnotes under Table 4)

Table 4. Concentrations of (-)-sarin (ng/ml) and total blood BuChE (nM) in blood of individual anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. administration of a dose of (±)-sarin corresponding with 2 LD50 (48 µg/kg). Guinea pigs were injected (i.m.) with HuBuChE 24 h before (±)-sarin administration.

	GBH 1[a]		GBH 2		GBH 3		GBH 4[b]		GBH 5	
Weight (g)	535		695		640		685		790	
HuBuChE dose (nmol)	86		133		145		108		136	
Time (min)	(-)-sarin	BuChE	(-)-sarin	BuChE	(-)-sarin	BuChE	(-)-sarin	BuChE	(-)-sarin	BuChE
0	0	625	0	875	0	1067	0	457	0	609
1	2.03	125	x	370	0.11	262	3.51	70	0.18	166
2	1.49	100	0.11	465	0.13	221	2.59	20	0.19	161
4	1.20	66	x	311	0.19	210	0.95	14	0.17	143
10	0.13	70	0.11	256	0.37	240	0.18	4	0.14	115
20	0.10	108	0.08	225	0.20	220	0.15	19	0.22	158
40	0.063	66	0.08	193	0.30	260	0.21	33	x	155
60	0.070	90	0.07	193	0.09	256	0.18	21	x	168
120	0.160	90	0.05	211	0.05	230	n.s.	n.s.	n.s.	n.s.

x= analysis failed, n.s.= not sampled

[a] concentration-time course was fitted to [(-)-sarin] = $a*e^{(-b*t)} + c$, with a=2.77, b=0.312, c=0.077, r=0.978

[b] concentration-time course was fitted to [(-)-sarin] = $a*e^{(-b*t)} + c$, with a=5.66, b=0.478, c=0.166, r=0.996

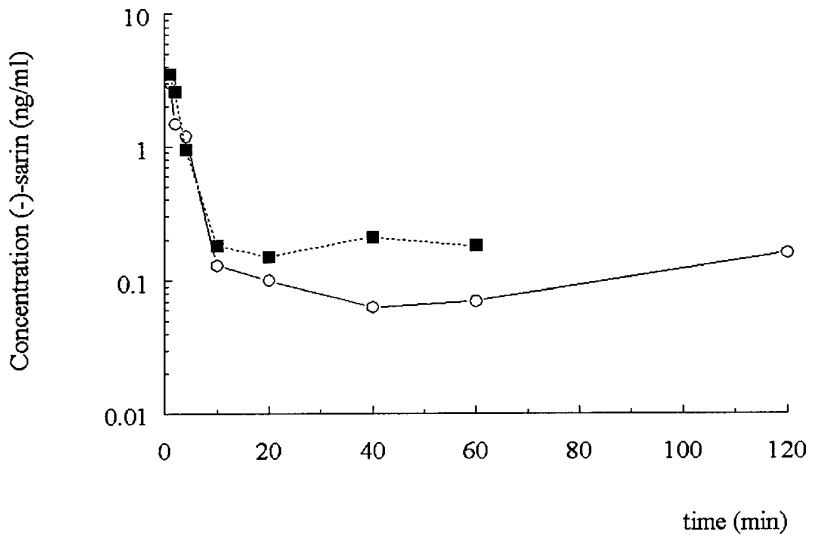

Figure 3. Concentration-time courses of (-)-sarin (ng/ml) in blood of anesthetized, atropinized, mechanically ventilated and HuBuChE-pretreated guinea pigs after i.v. administration of a (±)-sarin dose of 48 µg/kg, which corresponds with 2 LD50. ○ = guinea pig GBH 1, ■ = guinea pig GBH 4.

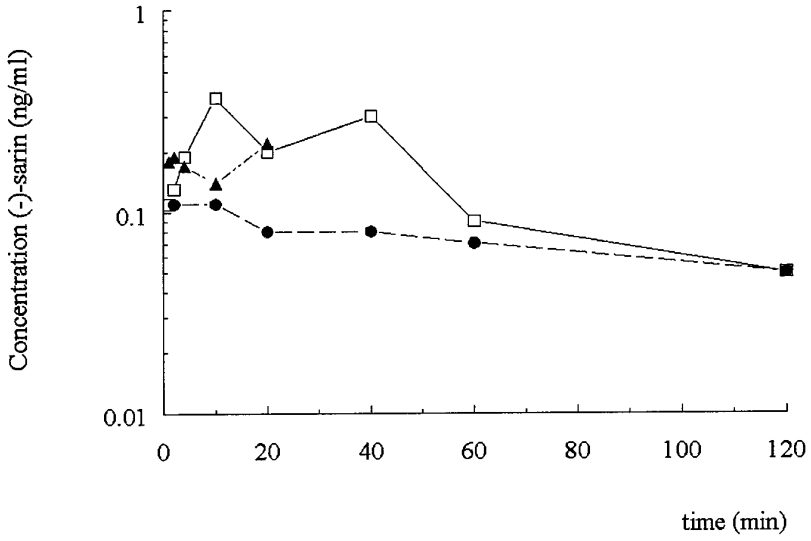

Figure 4. Concentration-time courses of (-)-sarin (ng/ml) in blood of anesthetized, atropinized, mechanically ventilated and HuBuChE-pretreated guinea pigs after i.v. administration of a (±)-sarin dose of 48 µg/kg, which corresponds with 2 LD50. ● = guinea pig GBH 2, □ = guinea pig GBH 3, ▲ = guinea pig GBH 5. Note: the scale of the Y-axis differs from that in Figure 3.

The blood samples taken at the final time point were also subjected to our fluoride-induced reactivation method (Polhuijs *et al.*, 1997). Incubation with a high concentration of fluoride ions under acidic conditions releases the phosphyl moiety covalently bound to BuChE, thus (re)generating (±)-sarin that can be measured gas chromatografically. The results are presented in Table 5.

Table 5. Initial concentrations of BuCHE (nM), AUC (0-20') (ng.min.ml^{-1}), and concentration of fluoride-induced reactivated (±)-sarin in the final blood samples of the toxicokinetic experiments with (±)-sarin (2 LD50, i.v.) in HuBuChE-pretreated and naive guinea pigs.

Experiment	Initial BuChE (nM)	AUC (0-20') (ng.min.ml^{-1})	F reactivated (±)-sarin (nM)	F reactivated (±)-sarin (ng/ml)
GBH 1	625	10.4	n.m.	n.m.
GBH 2	875	2.5	n.m.	n.m.
GBH 3	1067	5.4	1221	171
GBH 4	457	15.2	1250	175
GBH 5	609	3.9	1014	142
GB 1 to GB 4	20 ± 10 (n=4)	19.9 (n=4)	221±133 (n=4)	31±18 (n=4)

n.m. = not measured

The concentration of (±)-sarin that can be generated from binding sites in the final blood samples by fluoride-induced reactivation is much higher in the HuBuChE-pretreated animals than in naive animals.
The data in Table 5 also show that the AUC (0-20') for (-)-sarin in blood is lower in the HuBuChE-pretreated guinea pigs than in naive animals. This phenomenon is most pronounced in

experiments GBH 2, 3 and 5, as was anticipated on the basis of the low (-)-sarin concentrations in the blood of these animals very shortly after i.v. administration of (±)-sarin.
Another interesting observation from the data in Table 5 is that the concentration in nM of fluoride-generated (±)-sarin from the final blood samples exceeds the initial concentrations of BuChE in these animals. This indicates the presence of other binding sites than (Hu)BuChE in the blood of the guinea pig, such as carboxylesterases.

III.3 TOXICOKINETICS OF (±)-SARIN STEREOISOMERS IN ANESTHETIZED, AND ATROPINIZED GUINEA PIGS PRETREATED WITH HuBuChE DURING AND AFTER 2-MIN NOSE-ONLY EXPOSURE TO (±)-SARIN VAPOR IN AIR (T.O. 8)

Within the context of cooperative agreement DAMD17-90-Z-0034 we have determined the 24-h LC50 of (±)-sarin in anesthetized and atropinized guinea pigs for an 8-min nose-only exposure (Benschop and Van Helden, 1993). The LC50 was established to be 47 $mg.m^{-3}$ (95-% confidence intervals 44-50 $mg.m^{-3}$). For an 8-min exposure this corresponds with an LCt50-value of 376 $mg.min.m^{-3}$. For the purpose of the current study we proposed to expose guinea pigs to 2 LCt50 in 2 min. Assuming that Haber's rule would apply, i.e., that any combination of concentration and exposure time would yield an identical biological response at the same C*t-value, this would imply nose-only exposure of the guinea pigs to a concentration of (±)-sarin vapor in air of 376 $mg.m^{-3}$ for 2 min. Due to the high volatility of (±)-sarin, generating such a concentration was no problem at all. Upon exposure of three HuBuChE-pretreated guinea pigs to this high concentration in order to study the toxicokinetics, two out of three animals died before the planned end point of the experiment. The results are presented in Table 6 and Figure 5.

According to our proposal, the guinea pigs were to be pretreated with a dose of HuBuChE corresponding with 0.25 times the molar dose of 2 LCt50 of C(±)P(±)-soman (8-min exposure). The required dose of HuBuChE was estimated as follows: a 500-g guinea pig breathes 83 ml of air per min (Trap, 2002). The LCt50 (8-min) of C(±)P(±)-soman was determined to be 480 $mg.min.m^{-3}$. For exposure to 2 LCt50 in 2 min, the guinea pig has to be exposed to a concentration of C(±)P(±)-soman vapor in air of 480 $ng.ml^{-1}$ for 2 min. Consequently, the guinea pig inhales 480 $(ng.ml^{-1})$*2 (min)*83 $(ml.min^{-1})$= 79680 ng of C(±)P(±)-soman. Assuming a retention of the agent of ca. 50 % (Trap, 2002) means that the internal dose would be 39840 ng, which corresponds with 218 nmol. Consequently, the dose of HuBuChE to be administered for pretreatment against a respiratory nerve agent challenge should be 0.25* 218 nmol = 54 nmol (≡ 3240 U). Guinea pigs GBH 9, 10 and 11 received doses of HuBuChE that came pretty close to 54 nmol (see Table 6). However, since the body weight of these animals was in the range of 658-685 g instead of 500 g, they should have been pretreated with 71-74 nmol of HuBuChE. Actually, these animals were pretreated with a dose corresponding with ca. 5000 U/kg. For practical purposes it was decided to continue pretreatment with a HuBuChE dose of *ca.* 5000 U/kg throughout the inhalation experiments.

Table 6. Concentrations of (±)-sarin stereoisomers (ng/ml) and total blood BuChE (nM) in blood of anesthetized, atropinized and HuBuChE-pretreated guinea pigs during and after a 2-min nose-only exposure to 376 $mg.m^{-3}$ of (±)-sarin vapor in air.

Animal #	GBH 9		GBH 10[a]			GBH 11[b]		
Weight (g)	658		683			685		
HuBuChE dose (nmol)	55.6		56.9			56.7		
Time (min)	(-)-sarin	BuChE	(+)-sarin	(-)-sarin	BuChE	(+)-sarin	(-)-sarin	BuChE
0	n.d.	298	n.d.	n.d.	386	n.d.	n.d.	361
2	0.033	286	n.d.	27.3	96	0.97	35.3	37
4	0.087	208	2.7	25.6	80	2.16	25.3	35
10	0.123	97	0.44	5.83	78	0.023	1.12	35
20	0.060	88	0.024	0.118	10	0.016	0.131	12
40	0.228	97	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
60	0.225	105	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

n.d. = not detectable; n.s. = not sampled

[a] Animal died shortly after taken 20-min sample

[b] Animal died around 30 min

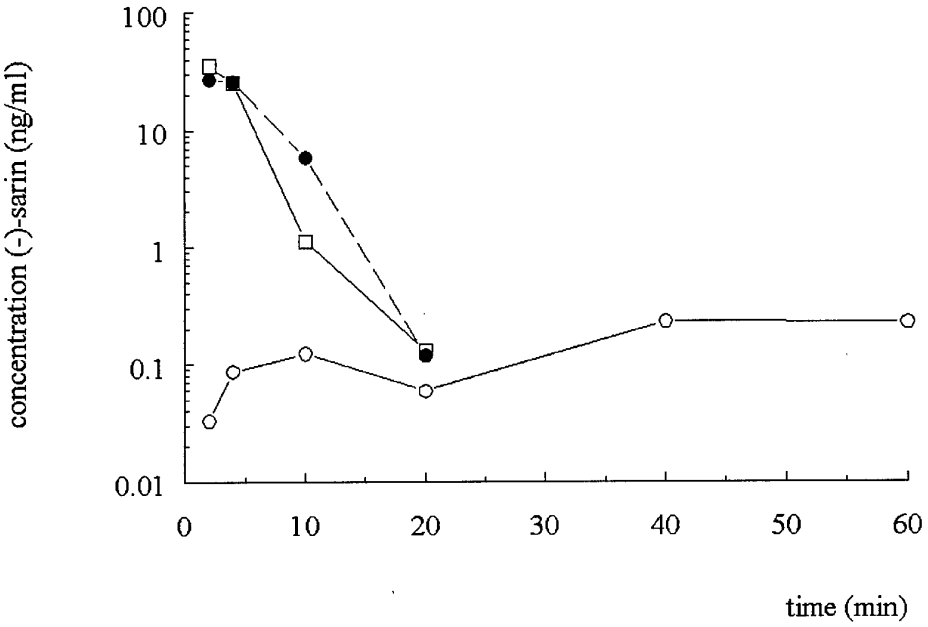

Figure 5. Concentration-time courses of (-)-sarin (ng/ml) in blood of anesthetized, atropinized, and HuBuChE-pretreated guinea pigs during and after a 2-min nose-only exposure to 376 $mg.m^{-3}$ of (±)-sarin vapor in air. ○ = guinea pig GBH 9, ● = guinea pig GBH 10, □ = guinea pig GBH 11.

The only animal that survived the 60-min experiment, GBH 9, was breathing very shallow and irregular in comparison with the other two animals. This implies that GBH 9 inhaled less of the (±)-sarin vapor during the 2-min exposure than GBH 10 and GBH 11, which provides for a tentative explanation why this animal survived the experiment. The observed lower concentrations of (-)-sarin in animal GBH 9 are in agreement with this hypothesis. It is interesting to note that in animals GBH 10 and 11 (+)-sarin could be detected in blood after the nose-only exposure to (±)-sarin. In previously performed inhalation studies involving nose-only exposure to 0.4 and 0.8 LCt50 (±)-sarin in 8 min, the (+)-sarin isomer was not detectable in the blood samples (Benschop and Van Helden, 1993).

The fact that two out three animals died within 30 min after exposure to 376 $mg.m^{-3}$ of (±)-sarin for 2 min, suggests that Haber's rule is not applicable under these conditions. Since we felt it would not be feasible to perform a toxicokinetic study of a reasonable time span (60 min) in this way, unless we would be willing to use considerably more animals than planned, we decided to lower the (±)-sarin vapor concentration to 200 $mg.m^{-3}$.
Both naive and HuBuChE-pretreated animals were nose-only exposed to this concentration for 2 min, and blood samples were drawn up to 60 min after the start of the exposure for analysis of the concentrations of (±)-sarin stereoisomers and BuChE activity. The results are presented in Tables 7 and 8, and Figures 6 and 7.

Table 7. Concentrations of (±)-sarin stereoisomers (ng/ml) in blood of anesthetized and atropinized guinea pigs during and after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of (±)-sarin vapor in air.

Animal #	GBH 12		GBH 15		GBH 16		GBH 18		GBH 21	
Weight (g)	713		617		633		768		668	
Time (min)	(+)-sarin	(-)-sarin	(+)-sarin	(-)-sarin	(+)-sarin	(-)-sarin	(+)-sarin	(-)-sarin	(+)-sarin	(-)-sarin
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	0.025	0.22	n.d.	0.48	n.d.	0.11	0.56	16.4	2.26	19.7
4	0.018	0.51	0.058	4.64	n.d.	0.48	0.58	6.14	0.091	6.14
10	n.d.	0.14	n.d.	0.81	n.d.	0.28	n.d.	0.62	0.078	1.40
20	n.d.	0.093	n.d.	0.31	n.d.	0.092	n.d.	0.12	n.d.	0.20
40	n.d.	0.16	n.d.	0.16	n.d.	0.23	n.d.	0.19	n.d.	0.15
60	n.d.	0.16	n.d.	0.13	n.d.	0.27	n.d.	0.25	n.d.	0.19

n.d. = not detectable; n.s. = not sampled

Table 8. Concentrations of (±)-sarin stereoisomers (ng/ml) and total blood BuChE (nM) in blood of anesthetized, atropinized and HuBuChE-pretreated guinea pigs during and after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of (±)-sarin vapor in air.

Animal #	GBH 13			GBH 14			GBH 17		GBH 19			GBH 20		
Weigth (g)	536			540			674		768			648		
HuBuChE dose (nmol)	42.6			45			49.4		65			65		
Time (min)	(+)-sarin	(-)-sarin	BuChE	(+)-sarin	(-)-sarin	BuChE	(-)-sarin	BuChE	(+)-sarin	(-)-sarin	BuChE	(+)-sarin	(-)-sarin	BuChE
0	n.d.	n.d.	296	n.d.	n.d.	302	n.d.	321	n.d.	n.d.	412	n.d.	n.d.	437
2	n.d.	n.d.	140	0.29	1.38	171	0.075	171	0.071	0.53	191	0.67	1.99	101
4	n.d.	n.d.	260	n.d.	0.33	109	0.23	120	0.17	0.62	61	0.016	3.06	17
10	n.d.	0.047	102	n.d.	0.046	25	0.17	102	0.14	0.31	105	n.d.	0.62	28
20	n.d.	0.079	137	n.d.	0.011	41	0.13	58	n.d.	0.13	79	n.d.	0.080	26
40	0.06	0.28	93	n.d.	0.007	59	0.27	40	n.d.	0.12	66	n.d.	0.12	26
60	*.	*	74	n.d.	0.06	31	0.29	60	n.d.	0.18	73	n.d.	0.12	26

n.d. = not detectable; n.s. = not sampled

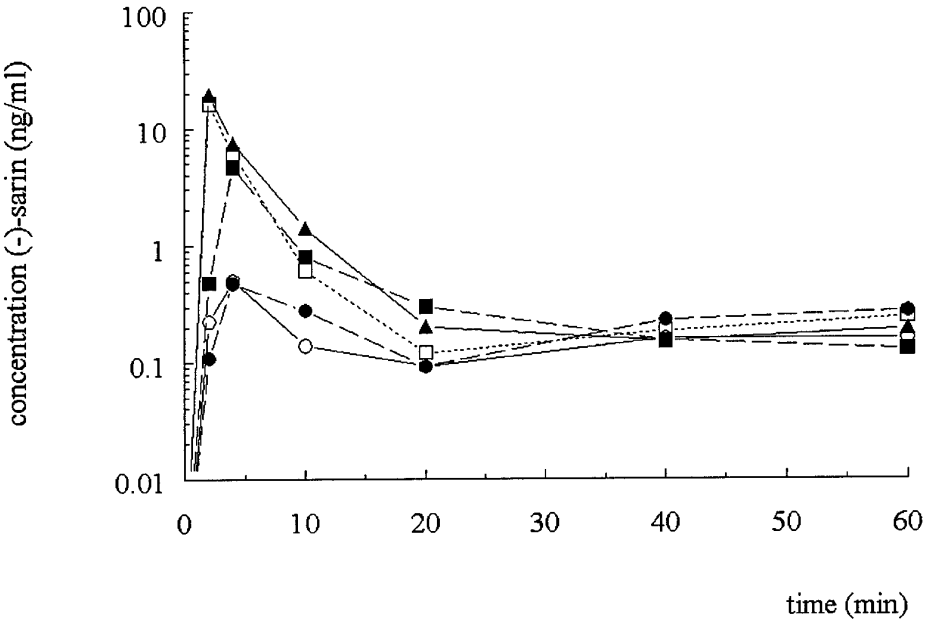

Figure 6. Concentration-time courses of (-)-sarin (ng/ml) in blood of anesthetized and atropinized guinea pigs during and after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of (±)-sarin vapor in air. ○ = guinea pig GBH 12, ■ - guinea pig GBH 15, ● = guinea pig GBH 16, □ = guinea pig GBH 18, ▲ = guinea pig GBH 21.

From the data presented in Table 7 and Figure 6 it is clear that the concentration of (-)-sarin increases during the 2-min exposure, and in some animals (GBH 12, GBH 15, GBH 16) continues to increase in the 2 min after ending the exposure. Such an increase in blood concentration of (-)-sarin after termination of the exposure was not observed for 8-min exposure to 0.4 and 0.8 LCt50 (±)-sarin (Benschop and Van Helden, 1993). Furthermore, it is remarkable that in most of the animals the (+)-sarin isomer is measurable in the first few minutes after ending the 2-min exposure, which - as already mentioned above - was not the case in the 8-min exposure studies.

The data presented in Table 7 and Figure 6 also show that there is more than a tenfold difference between the highest and the lowest curves measured in these five animals, at least in the time period up to 10-20 min. After that, all (-)-sarin concentrations are in the same range. Since the generated (±)-sarin vapor concentrations were all within the range of 200 ± 20 $mg.m^{-3}$, the variation between the curves is likely the result of differences in respiratory minute volumes and respiratory frequencies between the animals. Unfortunately, these parameters have not been measured.

Due to the large variation, it is probably not allowed to derive one mean concentration-time curve for these five experiments. It should be possible to derive toxicokinetic parameters for most of these concentration-time courses, but this has not been done yet. Also, the results of the fluoride-induced reactivation of covalently bound sarin in blood, as applied to the final blood samples of the toxicokinetic experiments are not yet available at this point in time. These results will be reported and discussed in the next Annual Report.

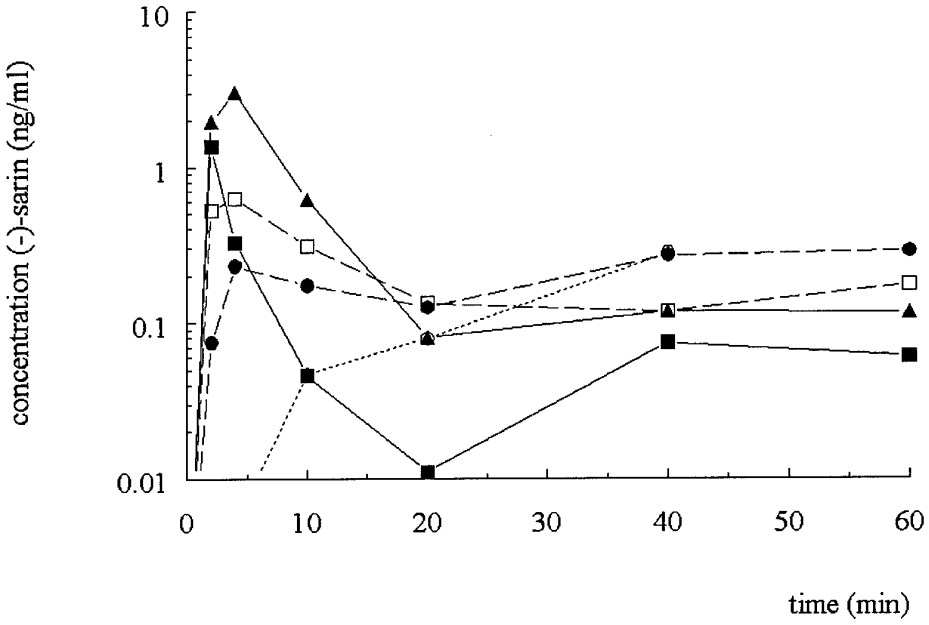

Figure 7. Concentration-time courses of (-)-sarin (ng/ml) in blood of anesthetized, atropinized and HuBuChE-pretreated guinea pigs during and after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of (±)-sarin vapor in air. ○ = guinea pig GBH 13, ■ = guinea pig GBH 14, ● = guinea pig GBH 17, □ = guinea pig GBH 19, ▲ = guinea pig GBH 20.

The data presented in Table 8 and Figure 7 show that also in the HuBuChE-pretreated animals the concentration of (-)-sarin increases during the 2-min exposure, and in some animals (GBH 17, GBH 19, GBH 20) continues to increase in the 2 min after ending the exposure. Furthermore, in most of the animals the (+)-sarin isomer is measurable in the first few minutes after ending the 2-min exposure. The BuChE activity is reduced considerably during the 2-min exposure and thereafter. As was also the case after intravenous administration of (±)-sarin to HuBuChE-pretreated animals, at the end of the toxicokinetic experiment there is still quite some residual BuChE activity left in the blood, whilst low concentrations of (-)-sarin are still circulating. Like in the naive animals, that there is more than a tenfold difference between the highest and the lowest curves measured in these five animals, in this case for the whole duration of the toxicokinetic experiment. Since the generated (±)-sarin vapor concentrations were all within the range of 200 ± 20 $mg.m^{-3}$, the observed variation between the curves is likely to be due to differences in respiratory minute volumes and respiratory frequencies between the animals, which we did not measure.

Although the concentration-time curves of the HuBuChE-pretreated animals tend to be somewhat lower than those measured in the naive animals, the difference is not dramatic. Presumably, the differences between the pretreated and non-pretreated groups will become more apparent on the basis of the AUC values and the concentrations of (±)-sarin (re)generated by fluoride-induced reactivation. However, these data are not available yet. These results, as well as those of the toxicokinetic evaluation of the concentration-time courses measured in the individual HuBuChE-pretreated guinea pigs, will be reported and discussed in the next Annual Report.

III.4 DETERMINATION OF THE RATE CONSTANT OF INHIBITION OF HuBuChE BY THE STEREOISOMERS OF THE NERVE AGENTS C(±)P(±)-SOMAN, (±)-SARIN AND (±)-VX (T.O.s 14-17)

The concentration of HuBuChE can be expressed as units (U)/ml in which 1 U hydrolyses 1 µmol of butyrylthiocholine in 50 mM phosphate buffer per min at pH 8.0 and 25 °C. The concentration of a solution of HuBuChE as U/ml was evaluated after determining the BuChE activity under these conditions, using the molar extinction coefficient of 2-nitro-5-mercapto-benzoic acid, i.e., the reaction product of which the extinction is measured in the activity assay. The molar extinction coefficient of 2-nitro-5-mercapto-benzoic acid was determined to be $1.396 * 10^4$. It is known from literature (Grunwald *et al.*, 1997) that 1 nmol of HuBuChE active sites corresponds with 60 units (U) of the enzyme. Hence, the molar concentrations of the HuBuChE active sites could subsequently be calculated from the enzyme concentration expressed as U/ml. This molar concentration of the enzyme should be known, in addition to that of the inhibitor in order to determine the rate constants of inhibition for the potent inhibitors (±)-sarin, (±)-VX and C(±)P(±)-soman.

The results obtained from reactions of the racemic mixtures of (±)-sarin and (±)-VX with HuBuChE did not completely obey the kinetics for a reaction of the enzyme with the two stereoisomers of the nerve agents reacting at the same rate, nor kinetics for reaction of the enzyme with only one stereoisomer due to high stereoselectivity. It was concluded from these results that HuBuChE shows a relatively low selectivity for the stereoisomers of these two organophosphates.

Values for the rate constants of inhibition of HuBuChE by the two stereoisomers of (±)-sarin and (±)-VX were obtained from a series of experiments in which inhibition of the enzyme by the racemic mixture of the organophosphate was followed with time. In these experiments, enzyme and inhibitor concentrations were only slightly different. The rate constants for the two parallel reactions proceeding with the two stereoisomers were evaluated by introducing a parameter θ, defined as $\theta = \int [E]\, dt$, in which [E] and t are enzyme concentration and reaction time, respectively, analogously to a procedure described by French (1950) (see Appendix 1). The calculated rate constants are presented in Tables 9 and 10.

Table 9. Rate constants for inhibition (k, $M^{-1}.min^{-1}$) of HuBuChE by the two stereoisomers of (±)-sarin at pH 7.4 and 38 °C; the initial concentrations of enzyme and inhibitor in the inhibition reactions are also shown.

Run	HuBuChE (nM)	Sarin (nM)	k_1 ($M^{-1}.min^{-1}$)	k_2 ($M^{-1}.min^{-1}$)	k_1/k_2
1	12.6	12.0	$4.1 * 10^7$	$6.1 * 10^6$	6.7
2	11.8	10.5	$4.2 * 10^7$	$4.4 * 10^6$	9.7
3	12.8	10.5	$4.2 * 10^7$	$6.1 * 10^6$	7.0
Mean			$(4.2 \pm 0.1) * 10^7$	$(5.5 \pm 1.0) * 10^6$	7.6

Table 10. Rate constants for inhibition (k, $M^{-1}.min^{-1}$) of HuBuChE by the two stereoisomers of (±)-VX at pH 7.4 and 38 °C; the initial concentrations of enzyme and inhibitor in the inhibition reactions are also shown.

Run	HuBuChE (nM)	VX (nM)	k_1 ($M^{-1}.min^{-1}$)	k_2 ($M^{-1}.min^{-1}$)	k_1/k_2
1	11.0	21	$0.95 * 10^7$	$3.4 * 10^6$	2.8
2	11.1	18	$1.45 * 10^7$	$1.2 * 10^6$	12
3	10.9	15	$1.65 * 10^7$	$0.4 * 10^6$	40
4	10.4	10.5	$1.27 * 10^7$	$2.7 * 10^6$	4.7
5	10.6	9.7	$1.39 * 10^7$	$3.5 * 10^6$	4.0
6	5.7	9.7	$1.34 * 10^7$	$3.5 * 10^6$	3.8
Mean			$(1.3 \pm 0.2) * 10^7$	$(2.5 \pm 1.3) * 10^6$	5.5

The rate constants of inhibition of HuBuChE by the four stereoisomers of C(±)P(±)-soman cannot be deduced in the same way as it was accomplished with (±)-sarin and (±)-VX. Therefore, the individual isomers of soman were isolated from C(+)P(±)-soman and C(-)P(±)-soman in the usual manner by treatment with either α-chymotrypsin or rabbit serum (Benschop *et al.*, 1984).
The inhibition reactions were performed under first-order conditions with respect to both enzyme and soman stereoisomer, except for inhibition by C(+)P(+)-soman. This isomer was used in a sufficiently large excess over the enzyme in order to justify description of the reaction as a pseudo first-order process. The results obtained for the four stereoisomers are presented given in Tables 11-14.

Table 11. Rate constants for inhibition (k, $M^{-1}.min^{-1}$) of HuBuChE by C(-)P(+)-soman at pH 7.4 and 38 °C; the initial concentrations of enzyme and inhibitor in the inhibition reactions are also shown.

Run	HuBuChE (nM)	C(-)P(+)-soman (nM)	k ($M^{-1}.min^{-1}$)
1	3.57	13.0	$7.1 * 10^6$
2	3.75	11.8	$7.3 * 10^6$
3	3.57	10.1	$6.7 * 10^6$
4	3.55	8.8	$7.3 * 10^6$
5	3.57	7.1	$8.3 * 10^6$
Mean			$(7.4 \pm 0.6) * 10^6$

Table 12. Rate constants for inhibition (k, $M^{-1}.min^{-1}$) of HuBuChE by C(+)P(+)-soman at pH 7.4 and 38 °C; the initial concentrations of enzyme and inhibitor in the inhibition reactions are also shown.

Run	HuBuChE (nM)	C(+)P(+)-soman (nM)	k ($M^{-1}.min^{-1}$)
1	2.85	71	$2.2 * 10^6$
2	3.55	64	$2.3 * 10^6$
3	3.45	58	$2.0 * 10^6$
4	3.63	49	$2.3 * 10^6$
Mean			$(2.1 \pm 0.2) * 10^6$

Table 13. Rate constants for inhibition (k, $M^{-1}.min^{-1}$) of HuBuChE by C(+)P(-)-soman at pH 7.4 and 38 °C; the initial concentrations of enzyme and inhibitor in the inhibition reactions are also shown.

Run	HuBuChE (nM)	C(+)P(-)-soman (nM)	k ($M^{-1}.min^{-1}$)
1	1.01	0.96	$3.5 * 10^8$
2	0.98	0.96	$3.6 * 10^8$
3	1.11	0.95	$3.8 * 10^8$
Mean			$(3.6 \pm 0.2) * 10^8$

Table 14. Rate constants for inhibition (k, $M^{-1}.min^{-1}$) of HuBuChE by C(-)P(-)-soman at pH 7.4 and 38 °C; the initial concentrations of enzyme and inhibitor in the inhibition reactions are also shown.

Run	HuBuChE (nM)	C(-)P(-)-soman (nM)	k ($M^{-1}.min^{-1}$)
1	10.7	26.7	$1.26 * 10^7$
2	11.6	22.4	$1.31 * 10^7$
3	8.6	21.1	$1.06 * 10^7$
4	12.3	19.9	$1.20 * 10^7$
Mean			$(1.2 \pm 0.1) * 10^7$

IV. DISCUSSION

Time course of HuBuChE after i.m. administration

From Table 1 and Figure 1 it is clear that i.m. administration of HuBuChE leads to an important increase in the BuChE activity that can be measured in blood. As was expected, after i.m. administration of HuBuChE the BuChE activity gradually builds up, reaches a maximum, and then decreases again.

Allon *et al.* (1998) have also studied the time course of BuChE activity in guinea pigs after i.m. administration of HuBuChE. The dose they administered was approximately 5400-5900 U/kg, which resulted in a maximum BuChE activity in blood of *ca.* 34 U/ml. In our initial experiments, a 3 to 4-fold higher dose of HuBuChE was required to obtain such a BuChE-level, which seems strange. Since we did not perform experiments with intravenous administration of HuBuChE to guinea pigs, we cannot establish whether this can be attributed to a lower bioavailability of the enzyme than the 93 % reported by Allon *et al.* (1998). We did have a dispute with the people at Walter Reed Army Institute of Research about the specific acitivity of the HuBuChE preparation that we received: according to our measurements we only received half the BuChE activity that was declared. According to WRAIR this was due to the way we measured the activity. However, since we based the doses we administered to the animals on our own measurements this cannot explain the discrepancy with the findings of Allon *et al.* (1998).

Interestingly, in the toxicokinetic studies the administered doses resulted in relatively higher BuChE activity levels than in the experiments in which the time course of BuChE activity was determined. As can be derived from the data in Table 15, the ratio between the measured BuChE activity in blood at 24 h after administration of HuBuChE and the administered dose per kg body weight was as low as 1.8 ± 0.2 (SEM, n=3) in the experiments to determine the time course of BuChE activity, 3.9 ± 0.3 (SEM, n=5) in the intravenous experiments with (±)-sarin, and 4.1 ± 0.2 (SEM, n=8) in the inhalation experiments with (±)-sarin. A different batch of HuBuChE was used in the toxicokinetic experiments than in the establishment of the time course of BuChE activity in blood after i.m. administration of the enzyme.

Table 15. Administered HuBuChE doses (U/kg body weight), measured BuChE activities (U/ml blood) at 24 h after administration, and the ratio of this measured activity and the administered dose, for all of the experiments performed so far in guinea pigs. For comparison, data calculated from Allon *et al.* (1998) are included.

Experiment	HuBuChE dose (U/kg)	BuChE activity at 24 h (U/ml blood)	Activity to dose ratio
Allon *et al.* (1998)	5500-5800	34 ± 6 (SEM)	$5.9\text{-}6.2*10^{-3}$
Guinea pig #1 (no sarin)	20700	34.8	$1.7*10^{-3}$
Guinea pig #2 (no sarin)	20700	33.4	$1.6*10^{-3}$
Guinea pig #3 (no sarin)	10500	22.4	$2.1*10^{-3}$
GBH 1 (sarin i.v.)	9645	37.5	$3.9*10^{-3}$
GBH 2 (sarin i.v.)	11482	52.5	$4.6*10^{-3}$
GBH 3 (sarin i.v.)	13594	64.0	$4.7*10^{-3}$
GBH 4 (sarin i.v.)	9460	27.4	$2.9*10^{-3}$
GBH 5 (sarin i.v.)	10329	36.5	$3.5*10^{-3}$
GBH 9 (sarin nose-only)	5073	17.9	$3.5*10^{-3}$
GBH 10 (sarin nose-only)	5000	23.2	$4.6*10^{-3}$
GBH 11 (sarin nose-only)	4964	21.7	$4.4*10^{-3}$
GBH 13 (sarin nose-only)	4767	17.8	$3.7*10^{-3}$
GBH 14 (sarin nose-only)	5000	18.1	$3.6*10^{-3}$
GBH 17 (sarin nose-only)	4399	19.3	$3.8*10^{-3}$
GBH 19 (sarin nose-only)	5078	24.7	$4.9*10^{-3}$
GBH 20 (sarin nose-only)	6018	26.2	$4.4*10^{-3}$

Obviously, in the toxicokinetic experiments the BuChE activities we measured are closer to those reported by Allon *et al.* (1998), albeit that these authors have accomplished a 1.5-fold higher activity to dose ratio of $6 \pm 1 * 10^{-3}$.
Allon *et al.* (1998) do not specify the toxicokinetic parameters for the i.m. administration of HuBuChE to guinea pigs. Therefore we cannot compare the rate constants for absorption and elimination that we estimated from our curves with their values.
At the 2002 Medical Chemical Defense Bioscience Review (Hunt Valley, MD) we discussed with Dr. Yacov Ashani (Israel Institute for Biological Research (IIBR), Ness Ziona, Israel) the discrepancy in BuChE activities after HuBuChE administration as measured at IIBR and TNO-PML. As outlined above, this discrepancy is no longer as large as it was initially. Nevertheless, we will perform some additional experiments, in which we will follow the BuChE activity in blood after i.m. administration over a longer period of time. Furthermore, we are considering to perform some experiments in which HuBuChE will be administered i.v., so that we can calculate the bioavailability of the i.m. administration.

Influence of HuBuChE pretreatment of guinea pigs on the intravenous toxicokinetics of (±)-sarin
Within the context of cooperative agreement DAMD17-90-Z-0034 we have studied the intravenous toxicokinetics of (±)-sarin in the anesthetized, atropinized and mechanically verntilated guinea pig at a dose corresponding with 0.8 LD50 (19.2 μg/kg) (Benschop and Van Helden, 1993). The intravenous toxicokinetics were mainly studied as an 'equitoxic' reference for the inhalation toxicokinetics at 0.8 LCt50 in an 8-min exposure. When we conceived the proposal for the current study on the effect of pretreatment with HuBuChE on the toxicokinetics of nerve agents we intended to use the previously measured toxicokinetic curve for 0.8 LD50 (±)-sarin in the naive guinea pig as a reference for the curve of 2 LD50 (±)-sarin in the HuBuChE-pretreated guinea pig, assuming a dramatic effect of HuBuChE-pretreatment on the toxicokinetics. When performing the experiments, the acceleration of the toxicokinetics appeared not to be that dramatic. As a result, we decided to perform a limited study on the intravenous toxicokinetics of (±)-sarin for a dose corresponding with 2 LD50 in naive guinea pigs to provide for an adequate reference for the experiments in HuBuChE-pretreated animals.
As was the case for an intravenous dose corresponding with 0.8 LD50, the (+)-isomer of sarin was not detectable in the blood of the guinea pigs after an i.v. bolus of a dose corresponding with 2 LD50. The half-life of distribution appears to be 0.15 min after 0.8 LD50 and 1.4 min after 2 LD50, whereas the terminal half-lives resulting from these doses are 58 and 140 min, respectively. The calculated AUC-values are 15.3 $ng.min.ml^{-1}$ for 0.8 LD50 and 228 $ng.min.ml^{-1}$ for 2 LD50, indicating non-linearity of the toxicokinetics with the dose, as was also observed for 0.8 and 2 LD50 of C(±)P(±)-soman (Benschop and Van Helden, 1993).
Previously, we already concluded that (-)-sarin is more persistent in the guinea pig than C(±)P(-)-soman at an i.v. dose corresponding with 0.8 LD50 (Benschop and Van Helden, 1993). This is also true for an i.v. dose of (±)-sarin corresponding with 2 LD50.
In Figure 8 the measured toxicokinetic curves in naive guinea pigs and HuBuChE-pretreated animals are shown. As we feel it is not justified to use an average of the data points measured in HuBuChE-pretreated animals, the concentration-time courses for the individual animals are shown, which makes this figure very busy. It shows however that the difference between the mean concentration-time course in naive animals (black diamond markers, thick black line, error bars) does not differ dramatically from some of the curves measured in pretreated animals. In particular the curves measured in animals GBH 1 and GBH 4 could have been just as well within the lower end variation of the mean curve in naive animals.
From the data in Table 4, there appears to be some correlation between the BuChE activity in blood just before administration of the nerve agent and the disappearance rate of (-)-sarin from the blood. In animals GBH 2 and GBH 3, the concentrations of (-)-sarin are very low almost immediately after the i.v. bolus, whereas in animal GBH 4, which had the lowest BuChE activity in blood of the five animals studied, the (-)-sarin concentrations are considerably higher immediately after administration.

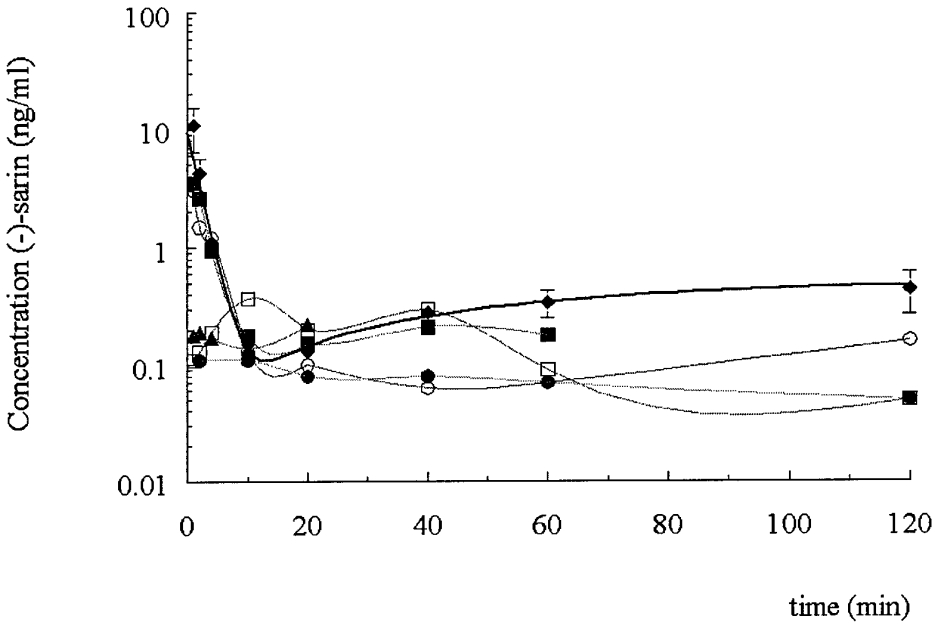

Figure 8. Mean concentration-time course of (-)-sarin (♦, ± SEM, n=4) in blood of anesthetized, atropinized and mechanically ventilated guinea pigs after i.v. administration of 2 LD50 (±)-sarin, as well as concentration-time courses of (-)-sarin in individual HuBuChE-pretreated animals (○, ●, □, ■, ▲) after i.v. administration of this dose.

It seems clear that the effect of HuBuChE on the toxicokinetics is most pronounced in the first minutes after administration of (±)-sarin. This observation is supported by the significant difference (student t-test, $p<0.05$) between the AUC values calculated from 0 to 20 min for the naive animals and the HuBuChE-pretreated animals.
It is remarkable that the concentrations of (-)-sarin remain rather constant at a relatively low level for a long period of time. Why these concentrations are not reduced faster by the still relatively high BuChE concentrations circulating in blood we cannot explain at this point.
Other proof for the effect of HuBuChE as a scavenger for (±)-sarin is found by applying the fluoride-induced reactivation method to the blood samples. Under acidic condition, a high concentration of fluoride ions is capable of reactivating the covalently bound phosphyl moiety from BuChE, thus regenerating (±)-sarin (racemic due to the high fluoride ion concentration). When applied to the final blood samples of the toxicokinetic experiments, significantly (student t-test $p<0.05$) higher concentrations of (±)-sarin are regenerated in blood samples taken from HuBuChE-pretreated animals (183 ± 18, SD_{n-1}, n=3) than in samples from naive animals (31 ± 18, SD_{n-1}, n=4), as apparent from Table 5.

Influence of HuBuChE pretreatment of guinea pigs on the inhalation toxicokinetics of (±)-sarin
In view of the rapid death of two out of three animals, Haber's rule appeared to be not applicable for converting the 24-h LC50 of (±)-sarin for an 8-min nose-only exposure to a value for a 2-min exposure. More likely the 'toxic load principle' (C^n*t=constant) should be used for this conversion. The value of 'n' is generally accepted to be *ca.* 1.5, as derived from a study by Cresthull *et al.* (1957) in which monkeys were exposed to G-agent vapors in 2 and 10 min. In

order not to waste more animals we decided more or less intuitively to lower the concentration of (±)-sarin vapor from 375 mg.m^{-3} to 200 mg.m^{-3}, which corresponds with a value of 'n' of 1.8. Applying n=1.5 would suggest exposure to 237 mg.m^{-3} for 2 min. Consequently, exposure to 200 mg.m^{-3} for 2 min may therefore be equivalent to somewhat less than 2 LCt50. Since the toxicokinetics are studied in both naive and HuBuChE-pretreated guinea pigs under these conditions, this deviation is not an insurmountable problem.

The dose of HuBuChE to be used for pretreatment in the inhalation toxicokinetic studies was calculated using values for the respiratory minute volume and the retention of C(±)P(±)-soman in anesthetized and atropinized guinea pigs that we actually measured (Trap, 2002). Allon *et al.* (1998) have used a different approach to calculate the desired dose of HuBuChE. In their experiments the guinea pigs were fully protected against *ca.* 2.5 inhaled LD50s of C(±)P(±)-soman at a dose of HuBuChE that is pretty close to the one we use. This means that the dose we calculated is in the right ballpark.

An interesting finding in the toxicokinetic study is that we were able to quantify (+)-sarin in blood after the 2-min exposure to concentrations of (±)-sarin vapor in air of 376 and 200 mg.m^{-3}, which was not the case during and after 8-min exposure to 19 and 38 mg.m^{-3}. (Benschop and Van Helden, 1993). Apparently the rate of absorption from the (upper) airways is higher than the rate of detoxification under these conditions. As anticipated, the (+)-isomer subsequently disappears more rapidly from the blood than the (-)-isomer, most likely due to hydrolysis. In inhalation experiments with C(±)P(±)-soman we did measure traces of one of the P(+)-isomers during 8-min exposure to 48 mg.m^{-3} of C(±)P(±)-soman vapor in air (corresponding with 0.8 LCt50) but oddly enough not during or after a 4-min exposure to a twofold higher concentration (Benschop and Van Helden, 1993).

Another interesting finding is the increase in (-)-sarin concentration in the first few minutes after ending the exposure to the nerve agent vapor, which we observed in the majority of the animals (HuBuChE-pretreated as well as naïve). This suggests that the absorption of the agent continues after ending the exposure, probably from a deposition site in the upper airways. This phenomenon was not observed for 8-min exposure to 0.4 and 0.8 LCt50 of (±)-sarin, neither for 8-min exposure to 0.4 and 0.8 LCt50, nor for a 4-min exposure to 0.8 LCt50 of C(±)P(±)-soman (Benschop and Van Helden, 1993). We did however observe that the concentrations of the P(-)-soman isomers remained higher after ending the nose-only exposure than after ending an equitoxic intravenous infusion, which also implies continuation of absorption after ending the exposure.

In nearly all of the intravenous and inhalation toxicokinetic curves, the concentration of (-)-sarin does not continue to decrease in the terminal phase of the toxicokinetics but in many cases appears to level off or even increase slightly at 1-2 h after administration or exposure. A similar phenomenon was observed for the toxicokinetics of 0.8 LD50 (i.v.) and 0.4 LCt50 (8-min nose-only exposure) of (±)-sarin (Benschop and Van Helden, 1993). Back then we consired the increasing (-)-sarin concentration near the end of the toxicokinetic curve to be an analytical artefact, resulting from the fact that the concentrations were approaching the detection limit of the analytical procedure. However, in view of the results obtained in the current study, this phenomenon can no longer be ignored. Interestingly, we hardly ever have seen the concentrations of either of the P(-)-isomers of soman to increase in the terminal phase. As most of the toxicokinetic experiments within the context of the current study are devoted to C(±)P(±)-soman, it will become apparent whether this phenomenon is actually associated with (±)-sarin and not with C(±)P(±)-soman. Tentative explanations for the levelling-off or increase of the (-)-sarin concentration in the terminal phase of the toxicokinetics are release from rather aspecific, non-covalent binding sites, or reactivation from covalent binding sites induced by endogenous fluoride ions. The apparent persistence of (-)-sarin may have important implications for the approaches to pretreatment and therapy of intoxications by this nerve agent.

In Figure 9 all ten curves measured for nose-only exposure to 200 mg.m^{-3} of (±)-sarin vapor in air are presented. Although this is a very busy graph, it is clear that there is no dramatic difference between the concentration-time courses of (-)-sarin in naive and HuBuChE-pretreated animals. In fact, the lower curves measured in naïve animals overlap with the higher curves measured in HubuChE-pretreated animals. In the terminal phase the concentrations of (-)-sarin

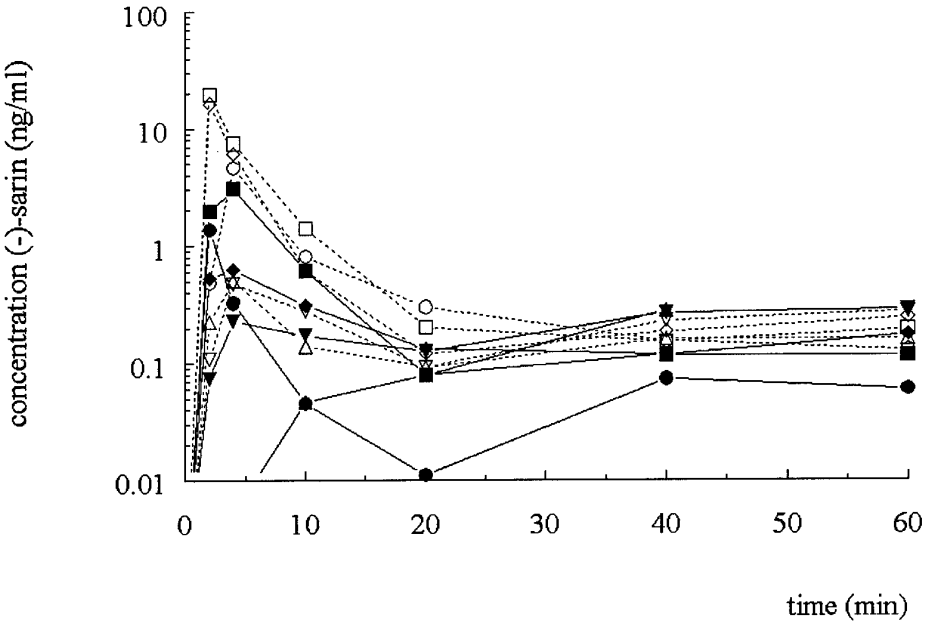

Figure 9. Concentration-time courses of (-)-sarin (ng/ml) in anesthetized and atropinized guinea pigs during and after a 2-min nose-only exposure to 200 $mg.m^{-3}$ (±)-sarin vapor in air. HuBuChE-pretreated animals: filled markers, solid lines; non-pretreated animals: open markers, dotted lines.

measured in the two groups of guinea pigs are very close together. Perhaps the AUC and the concentrations of fluoride-induced (re)generated (±)-sarin are better indicators for the efficacy of HuBuChE-pretreatment than the toxicokinetic curves themselves, as was observed for the intravenous toxicokinetics of (±)-sarin in naive and HuBuChE-pretreated animals. As stated in chapter III.3, these results are not available yet.

Rate constants of inhibition of HuBuChE for nerve agent stereoisomers

The molar concentration of HuBuChE, which is needed for studying the reaction of the enzyme with the potent inhibitors such as (±)-sarin, (±)-VX and C(±)P(±)-soman, could conveniently be derived from the activity of the enzyme by using the relation reported by Grunwald *et al.* (1997) between the activity of HuBuChE expressed as U/ml and its molar concentration.

From the inhibition studies it was concluded that the ratio between the anticholinesterase activities of the two stereoisomers of (±)-sarin and (±)-VX differs only slightly. These results are in correspondence with the general finding that BuChE shows only a minor stereoselectivity for potent chiral organophosphates, in contrast with the high stereoselectivity usually exhibited by AChE for such inhibitors.

The rate constants of inhibition for the two stereoisomers of (±)-sarin and (±)-VX could be evaluated from the reaction of the enzyme with a slightly different concentration of the racemic mixture of the organophosphate by using a kinetic analysis based on a method reported by French (1950). The results obtained for inhibition of HuBuChE are of the same order of magnitude as data previously obtained for horse serum BuChE (see Table 16).

Table 16. Rate constants of inhibition ($M^{-1}.min^{-1}$) of human and horse serum BuChE by the stereoisomers of (±)-sarin, (±)-VX and C(±)P(±)-soman .

Organophosphate	Enzyme source			
	Human		Horse	
Sarin	$4.2 * 10^7$ $5.5 * 10^6$	pH 7.4, 38 °C[a]	$6 * 10^6$ $6 * 10^6$	pH 7.7, 25 °C[b]
VX	$1.3 * 10^7$ $2.5 * 10^6$	pH 7.4, 38 °C[a]	$1.7 * 10^7$	pH 7.7, 25 °C[c]
Soman				
C(+)P(-)	$3.8 * 10^8$	pH 7.4, 38 °C[a]	$8.7 * 10^7$	pH 7.7, 25 °C[d]
C(-)P(-)	$1.2 * 10^7$		$6.4 * 10^6$	
C(-)P(+)	$7.4 * 10^6$		$6.4 * 10^6$	
C(+)P(+)	$2.1 * 10^6$		$2.9 * 10^5$	
C(+)P(-)	$4 * 10^7$	pH 7.5, 25 °C[e]		
C(-)P(-)	$5 * 10^6$			
C(-)P(+)	$6 * 10^6$			
C(+)P(+)	n.d.[f]			

[a] Present study; [b] Boter and Van Dijk (1969); [c] rate constant for racemic mixture, unpublished results of TNO Prins Maurits Laboratory; [d] Keijer and Wolring (1969); [e] Millard *et al.* (1998); [f] not determined

V. CONCLUSIONS

1. Intramuscular administration of HuBuChE to guinea pigs leads to a gradual build-up of BuChE activity in blood. The maximum activity is reached at 20-24 h after administration, after which this activity remains relatively constant for another 24 h.

2. The observed time course of BuChE activity in the blood of the guinea pig after intramuscular administration of HuBuChE is comparable to that reported by Allon *et al.* (1998), albeit that we needed a higher dose of HuBuChE to obtain the same BuChE activity in the blood of the guinea pig.

3. The optimal time point for studying the effect of HuBuChE-pretreatment on the toxicokinetics of nerve agents in guinea pigs was chosen as 24 h after i.m. bolus administration.

4. The influence of HuBuChE-pretreatment on the concentration-time course of (-)-sarin in anesthetized, atropinized, and mechanically ventilated guinea pigs after i.v. bolus administration of a dose of (±)-sarin corresponding with 2 LD50 is not as dramatic as anticipated. The effect is most pronounced in approximately the first 10 min after administration, after which low concentrations of (-)-sarin persist for several hours.

5. The area-under-the-curve (AUC) for the first 20 min after administration appears to be a good indicator for the efficacy of HuBuChE as a scavenger for (±)-sarin, as the AUC values for (-)-sarin in blood differ significantly between the naive and HuBuChE-pretreated animals after i.v. bolus administration of 2 LD50 (±)-sarin.

6. Fluoride-induced reactivation of the phosphyl moiety of (±)-sarin covalently bound to BuChE, followed by measurement of the generated amount of (±)-sarin using GLC, provides for a suitable method to demonstrate the efficacy of HuBuChE as a scavenger for (±)-sarin, as significantly different concentrations of (±)-sarin were generated from blood samples taken from naive and HuBuChE-pretreated animals at 120 min after i.v. bolus administration of 2 LD50 (±)-sarin.

7. Haber's rule is not applicable for conversion of the LC50 of (±)-sarin for an 8-min nose-only exposure to a value for 2-min exposure.

8. The influence of HuBuChE-pretreatment on the concentration-time course of (-)-sarin in anesthetized and atropinized guinea pigs during and after a 2-min nose-only exposure to 200 $mg.m^{-3}$ of (±)-sarin vapor in air is not as dramatic as anticipated.

9. In the terminal phase of the toxicokinetics the concentrations of (-)-sarin appear to level off or even increase. So far, this phenomenon seems only to occur with (±)-sarin, and not with C(±)P(±)-soman.

10. HuBuChE shows only a minor stereoselectivity for chiral organophosphates, in contrast with the high stereoselectivity exhibited by AChE towards these anticholinesterases.

11. The results obtained for inhibition of HuBuChE are of the same order of magnitude as data previously reported for horse serum BuChE.

LITERATURE CITED

Allon, N., Raveh, L., Gilat, E., Cohen, E., Grunwald, J., and Ashani, Y. (1998) Prophylaxis against soman inhalation toxicity in guinea pigs by pretreatment alone with human serum butyrylcholinesterase. *Toxicol. Sci.* **43**, 121-128.

Ashani, Y., Shapira, S., Levy, D., Wolfe, A.D., Doctor, B.P., and Raveh, L. (1991) Butyrylcholinesterase and acetylcholinesterase prophylaxis against soman poisoning in mice. *Biochem. Pharmacol.* **41**, 37-41.

Ashani, Y., Grunwald, J., Raveh, L., Grauer, E., Brandeis, R., Marcus, D., Papier, Y., Kadar, T., Allon, N., Gilat, E., and Lerer, S. (1993) Cholinesterase prophylaxis against organophosphorus poisoning. Final Report Contract DAMD17-90-C-0033; AD-A277-096.

Benschop, H.P., and De Jong, L.P.A. (1988) Nerve agent stereoisomers: analysis, isolation, and toxicology. *Acc. Chem. Res.* **21**, 368-374.

Benschop, H.P., and De Jong, L.P.A. (1991) Toxicokinetics of soman: Species variation and stereospecificity in elimination pathways. *Neurosci. Biobehav. Rev.* **15**, 73-77.

Benschop, H.P. and Van Helden, H.P.M. (1993) Toxicokinetics of inhaled soman and sarin in guinea pigs. Final Report Grant DAMD17-90-Z-0034.

Benschop, H.P., Konings, C.A.G., Van Genderen, J., and De Jong, L.P.A. (1984) Isolation, anticholinesterase properties, and acute toxicity in mice of the four stereoisomers of the nerve agent soman. *Toxicol. Appl. Pharmacol.* **72**, 61-74.

Benschop, H.P., Bijleveld,. E.C., De Jong, L.P.A., Van der Wiel, H.J., and Van Helden, H.P.M. (1987) Toxicokinetics of the four stereoisomers of the nerve agent soman in atropinized rats – Influence of a soman simulator. *Toxicol. Appl. Pharmacol.* **90**, 490-500.

Benschop, H.P., De Jong, L.P.A., and Langenberg, J.P. (1995) Inhalation toxicokinetics of C(±)P(±)-soman and (±)-sarin in the guinea pig. In: *Enzymes of the Cholinesterase Family* (Quin, D.M., Balasubramian, A.S., Doctor, B.P., and Taylor, P., Eds). Plenum Press, pp. 361-368.

Benschop, H.P., Trap, H.C., Spruit, W.E.T., Van der Wiel, H.J., Langenberg, J.P. and De Jong, L.P.A. (1998) Low level nose-only exposure to the nerve agent soman: toxicokinetics of soman stereoisomers and cholinesterase inhibition in atropinized guinea pigs. *Toxicol. Appl. Pharmacol.* **153**, 179-185..

Black, R.M., and Harrison, J.M. (1996) The chemistry of organophosphorus chemical warfare agents, in: *The chemistry of organophosphorus compounds, Vol. 4* (Hartley, F.R., Ed.), Chapter 10. John Wiley & Sons Ltd, Chichester, pp. 781-840.

Boter, H.L., and Van Dijk, C. (1969) Stereospecificity of hydrolytic enzymes on reaction with asymmetric organophosphorus compounds. III. The inhibition of acetylcholinesterase and butyrylcholinesterase with enantiomeric forms of sarin. *Biochem. Pharmacol.* **18**, 2403-2407.

Brimfield, A.A., Hunter, Jr., K.W., Lenz, D.E., Benschop, H.P., Van Dijk, C., and De Jong, L.P.A. (1985) Structural and stereochemical specificity of mouse monoclonal antibodies to the organophosphorus cholinesterase inhibitor soman. *Mol.Pharmacol.* **28**, 32-39.

Cohen, J.A., and Warringa, M.G.P.J. (1957) Purification and properties of dialkylfluorophosphates. *Biochim. Biophys. Acta* **26**, 29-39.

Cresthull, P., Koon, W.S., and Oberst, F.W. (1957) Inhalation effects (incapacitation and mortality) for monkeys exposed to GA GB and GF vapors. CWLR 2179, U.S. Army Chemical Warfare Laboratories Technical Report, Army Chemical Center, MD.

De Jong, L.P.A. (1993) Determination of parameters for development of a physiologically based model for the toxicokinetics of C(±)P(±)-soman. Final Report for Cooperative Agreement DAMD17-92-V-2004.

De Jong, L.P.A. (1996) Physiologically based modeling of C(±)P(±)-soman toxicokinetics in guinea pig. Final Report for Cooperative Agreement DAMD17-95-2-5009.

De Jong, L.P.A., Sweeney, R.E., Maxwell, D.M., Langenberg, J.P., and Benschop, H.P. (2000) A physiologically based model for the toxicokinetics of C(±)P(±)-soman in the atropinized guinea pig and marmoset - Effect of scavengers and hydrolytic enzymes promoting elimination. In: *Proceedings of the 2000 U.S. Army Medical Defense Bioscience Review*, 4-6 June 2000, Hunt Valley (MD), pp. 98-107.

De La Hoz, D., Doctor, B.P., Ralston, J.S., Rush, R.S., and Wolfe, A.D. (1986) A simplified procedure for the purification of large quantities of fetal bovine serum acetylcholinesterase. *Life Sci.* **39**, 195-199.

Due, A.H., Trap, H.C., Van der Wiel, H.J., and Benschop, H.P. (1993) The effect of pretreatment with CBDP on the toxicokinetics of soman stereoisomers in rats and guinea pigs. *Arch. Toxicol.* **67**, 706-711.

Due, A.H., Trap, H.C., Langenberg, J.P., and Benschop, H.P. (1994) Toxicokinetics of soman stereoisomers after subcutaneous administration to atropinized guinea pigs. *Arch. Toxicol.* **68**, 60-63.

Fleisher, J.H., Harris, L.W. (1965) Dealkylation as a mechanism for ageing of cholinesterase after poisoning with pinacolyl methylphosphonofluoridate. Biochem. Pharmacol. **14**, 641-650.

French, D. (1950) Kinetic analysis of consecutive irreversible second order reactions. J. Am. Chem. Soc. **72**, 4806-4807.

Grunwald, J., Marcus, D., Papier, Y, Raveh, L., Pittel, Z., and Ashani, Y. (1997) Large-scale purification and long-term stability of human butyrylcholinesterase: a potential biocsavenger drug, J. Biochem. Biophys. Methods **34**, 123-135.

Harris, L.W., Braswell, L.M., Fleisher, J.H., and Cliff, W.J. (1964) Metabolites of pinacolyl methylphosphonofluoridate (soman) after enzymatic hydrolysis *in vitro*. Biochem. Pharmacol. **13**, 1129-1136.

Johnson, C.D., and Russell, R.L. (1975) A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations. *Anal. Biochem.* **64**, 229-238.

Keijer, J.H., and Wolring, G.Z. (1969) Stereospecific aging of phosphonylated cholinesterases. *Biochim. Biophys. Acta* **185**, 465-468.

Langenberg, J.P., Van Dijk, C., Sweeney, R.E., Maxwell, D.M., De Jong, L.P.A., and Benschop, H.P. (1997) Development of a physiologically based model for the toxicokinetics of C(±)P(±)-soman in the atropinized guinea pig. *Arch. Toxicol.* **71**, 320-331.

Langenberg, J.P., Spruit, W.E.T., Van der Wiel, H.J., Trap, H.C., Helmich, R.B., Bergers, W.W.A., Van Helden, H.P.M., and Benschop, H.P. (1998) Inhalation toxicokinetics of soman stereoisomers in the atropinized guinea pig with nose-only exposure to soman vapor. *Toxicol. Appl. Pharmacol.* **151**, 79-87.

Lenz, D.E., Brimfield, A.A., Hunter, Jr., K.W., Benschop, H.P., De Jong, L.P.A., Van Dijk, C., and Clow, T.R. (1984) Studies using a monoclonal antibody against soman. *Fundam. Appl. Toxicol.* **4**, S156-S164.

Marrs, T.C., Maynard R.L., and Sidell, F.R. (1996) Chemical Warfare Agents. Toxicology and Treatment. John Wiley & Sons Ltd, Chichester.

Maxwell, D.M., Castro, C.A., De La Hoz, D., Gentry, M.K., Gold, M.B., Solana, R.P., Wolfe, A.D., and Doctor, B.P. (1992) Protection of rhesus monkeys against soman and prevention of performance decrement by pretreatment with acetylcholinesterase. *Toxicol. Appl. Pharmacol.* **115**, 44-49.

Millard, C.B., Lockridge, O., and Broomfield, C.A. (1998) Organophosphorus acid anhydride hydrolase activity in human butyrylcholinesterase: synergy results in a somanase. *Biochemistry* **37**, 237-247.

Pearce, P., Passingham, S., Scott, L., Busker, R., and Van Xanten, N. (1999) A comparison of oximes, when combined with atropine and Avizafone, in the treatment of nerve agent poisoning in absence of pretreatment. Abstract Book of the Meeting of NATO TG004 in Brussels, March 22-26, 1999.

Polhuijs, M., Langenberg, J.P., and Benschop, H.P. (1997)New method for retrospective detection of exposure to organophosphorus anticholinesterases. Application to alleged sarin victims of Japanese terrorists. *Toxicol. Appl. Pharmacol.* **146**, 156-161.

Raveh, L., Grauer, E., Grunwald, J., Cohen, E., and Ashani, Y, (1997) The stoichiometry of protection against soman and VX toxicity in monkeys pretreated with human butyrylcholinesterase. *Toxicol. Appl. Pharmacol.* **145**, 43-53.

Spruit, W.E.T, Langenberg, J.P., Trap, H.C., Van der Wiel, H.J., Helmich, R.B., Van Helden, H.P.M., and Benschop, H.P. (2000) Intravenous and inhalation toxicokinetics of sarin stereoisomers in atropinized guinea pigs. *Toxicol. Appl. Pharmacol.* **169**, 249-254.

Trap, H.C. (2002), Unpublished results of TNO Prins Maurits Laboratory.

Van Helden, H.P.M., Langenberg, J.P., and Benschop, H.P. (2000) Low level exposure to GB vapor in air: diagnosis/dosimetry, lowest observable effect levels, performance-incapacitation, and possible delayed effects. Final Report for Grant Agreement DAMD17-97-1-7360.

Van der Schans, M.J., Langenberg, J.P., and Benschop, H.P. (2000) Toxicokinetics of O-ethyl S-(2-diisopropylaminoethyl) methylphosphonothioate [(±)-VX] in hairless guinea pigs and marmosets – Identification of metabolic pathways. Final Report for Cooperative Agreement DAMD17-97-2-7001.

Wolfe, A.D., Rush, R.S., Doctor, B.P., Koplovitz, I., and Jones, D. (1987) Acetylcholinesterase prophylaxis against organophosphate toxicity. *Fundam. Appl. Toxicol.* **9**, 266-270.

Wolfe, A.D., Blick, D.W., Murphy, M.R., Miller, S.A., Gentry, M.K., Hartgraves, S.L., and Doctor, B.P. (1992) Use of cholinesterase as pretreatment drugs for the protection of rhesus monkeys against soman toxicity. *Toxicol. Appl. Pharmacol.* **117**, 189-193.

TECHNICAL OBJECTIVES/STATEMENT OF WORK

Toxicokinetic studies

1. Determination of the time course of HuBuChE activity in blood of guinea pigs after i.m. administration of the enzyme. A dose of HuBuChE will be used which results in a total amount of the enzyme in blood that is 0.7 times the dose corresponding with 2 LD50 (i.v.) of C(±)P(±)-soman, just prior to nerve agent administration.

2. Determination of the time courses of C(±)P(±)-soman stereoisomers and of HuBuChE activity in blood of guinea pigs after pretreatment with HuBuChE (i.m.; dose as for item 1) and intoxication with an i.v. bolus dose corresponding with 2 LD50 of the agent.

3. Determination of the time courses of C(±)P(±)-soman stereoisomers and of HuBuChE activity in blood of guinea pigs after pretreatment with HuBuChE (i.m.; dose as for item 1) and intoxication with a second i.v. bolus dose corresponding with 2 LD50 of the agent given 90 min after the first dose.

4. Determination of the time courses of (±)-sarin stereoisomers and of HuBuChE activity in blood of guinea pigs after pretreatment with HuBuChE (i.m.; dose as for item 1) and intoxication with an i.v. bolus dose corresponding with 2 LD50 of the agent.

5. Determination of the time courses of (±)-VX stereoisomers and of HuBuChE activity in blood of hairless guinea pigs after pretreatment with HuBuChE (i.m.; dose as for item 1) and intoxication with an i.v. bolus dose corresponding with 2 LD50 of the agent.

6. Determination of the time courses of C(±)P(±)-soman stereoisomers and of HuBuChE activity in blood of guinea pigs pretreated with HuBuChE (i.m.) and intoxicated by nose-only exposure during 2 min to a dose corresponding to 2 LCt50 of the agent. A dose of HuBuChE will be used which results in a total amount of the enzyme in blood that is ca. 0.25 times the dose corresponding with 2 LCt50 of C(±)P(±)-soman in guinea pigs, just prior to nerve agent exposure.

7. Determination of the time courses of C(±)P(±)-soman stereoisomers and of HuBuChE activity in blood of guinea pigs pretreated with HuBuChE (i.m., dose as for item 6) and intoxicated by nose-only exposure during 300 min to a dose corresponding to 2 LCt50 of the agent.

8. Determination of the time courses of (±)-sarin stereoisomers and of HuBuChE activity in blood of guinea pigs pretreated with HuBuChE (i.m., dose as for item 6) and intoxicated by nose-only exposure during 2 min to a dose corresponding to 2 LCt50 of the agent.

9. Determination of the time courses of (±)-VX stereoisomers and of HuBuChE activity in blood of hairless guinea pigs pretreated with HuBuChE (i.m., dose as for item 1) and intoxicated with a percutaneous dose corresponding with 2 LD50 of the agent.

10. Determination of the time course of HuBuChE activity in blood of marmosets after i.m. administration of the enzyme. A dose of HuBuChE will be used which results in a total amount of the enzyme in blood that is *ca.* 0.5 times the dose corresponding with 2 LCt50 of C(±)P(±)-soman in marmosets, just prior to nerve agent exposure.

11. Construction of a nose-only exposure chamber for marmosets.

12. Determination of the time courses of C(±)P(±)-soman stereoisomers and of HuBuChE activity in blood of marmosets pretreated with HuBuChE (i.m., dose as for item 10) and intoxicated by a 2-min nose-only exposure to an (estimated) dose corresponding with 2 LCt50 of the agent.

13. Determination of the time courses of C(±)P(±)-soman stereoisomers in blood of marmosets intoxicated by a 2-min nose-only exposure to an (estimated) dose corresponding with 0.8 LCt50 of the agent.

Stereoselectivity of HuBuChE inhibition

14. Isolation of C(+)P(+)- and C(-)P(+)-soman by treatment of C(+)P(±)- and C(-)P(±)-soman, respectively, with α-chymotrypsin and isolation of C(+)P(-)- and C(-)P(-)-soman by treatment of C(+)P(±)- and C(-)P(±)-soman, respectively, with rabbit serum.

15. *In vitro* determination of the rate constants for inhibition of HuBuChE by the single stereoisomers of C(±)P(±)-soman at pH 7.5 and 38 °C.

16. *In vitro* determination of the rate constants for inhibition of HuBuChE by the stereoisomers of (±)-sarin at pH 7.5 and 38 °C from kinetic analysis of inhibition experiments performed with the racemic mixture.

17. *In vitro* determination of the rate constants for inhibition of HuBuChE by the stereoisomers of (±)-VX at pH 7.5 and 38 °C from kinetic analysis of inhibition experiments performed with the racemic mixture.

Binding in extravascular compartments

18. Determination of the HuBuChE activities in blood and in homogenates of lung, liver, kidney and brain from guinea pigs pretreated with HuBuChE (i.m., dose as for item 1). Blood and tissues will be removed at the point of time after enzyme administration at which the nerve agent is administered in the toxicokinetic experiments (items 2-5).

19. Determination of the HuBuChE activities, total concentrations of bound C(±)P(±)-^{14}C-soman and concentrations of C(±)P(±)-^{14}C-soman bound to HuBuChE in blood and in homogenates of lung, liver, kidney and brain from guinea pigs pretreated with HuBuChE (i.m., dose as for item 1) and intoxicated with a dose corresponding with 2 LD50 (i.v.) of C(±)P(±)-^{14}C-soman. Blood and tissues will be removed 1 min after C(±)P(±)-^{14}C-soman administration.

20. Determination of the HuBuChE activities, total concentrations of bound C(±)P(±)-^{14}C-soman and concentrations of C(±)P(±)-^{14}C-soman bound to HuBuChE in blood and in homogenates of lung, liver, kidney and brain from guinea pigs pretreated with HuBuChE (i.m., dose as for item 1) and intoxicated with a dose corresponding with 2 LD50 (i.v.) of C(±)P(±)-^{14}C-soman. Blood and tissues will be removed 90 min after C(±)P(±)-^{14}C-soman administration.

Physiologically based modeling

21. Development of a physiologically based model for the i.v. toxicokinetics of C(±)P(±)-soman in guinea pigs pretreated with HuBuChE (i.m.) by introduction into the model of data obtained in the present studies on the distribution of HuBuChE over various tissues (items 18-20) and on the rate constants for inhibition of the stereoisomers of C(±)P(±)-soman (item 15). Data obtained from toxicokinetic studies (item 2) will be used for validation of the model.

Appendix 1: Evaluation of the rate constants for inhibition of HuBuChE by two stereoisomers of an organophosphate showing a small difference in anticholinesterase activity, from reaction with its racemic mixture

Two parallel reactions proceed between enzyme (E) and the two stereoisomers of the organophosphate (L_+ and L)

$E + L_+ \rightarrow E\,L_+$
$E + L \rightarrow E\,L$

It is derived for these reactions that

$-d\,[E]/dt = k_1\,[E]\,[L_+] + k_2\,[E]\,[L]$ (1)
$-d\,[L_+]/dt = k_1\,[E]\,[L_+]$ (2)
$-d\,[L]/dt = k_2\,[E]\,[L]$ (3)

If parameter $\theta = \int_o^t [E]\,dt$ or $d\,\theta = [E]\,dt$ (4)

equations (1), (2) and (3) can be written as

$-d\,[E] = \{k_1\,[L_+] + k_2\,[L]\}\,d\,\theta$ (5)
$-d\,[L_+] = k_1\,[L_+]\,d\,\theta$ (6)
$-d\,[L] = k_2\,[L]\,d\,\theta$ (7)

After integration of equations (6) and (7) it is found that

$[L_+] = [L_{+,o}] \exp(-k_1\,\theta)$ (8)
$[L] = [L_{,o}] \exp(-k_2\,\theta)$ (9)

where $[L_{+,o}]$ and $[L_{,o}]$ are the initial concentrations of L_+ and L, respectively.

Substitution of equations (8) and (9) into equation (5) and integration of this equation leads to

$[E] = [E_o] + \tfrac{1}{2}\,[I_o]\,\{\exp(-k_1\,\theta) + \exp(-k_2\,\theta) - 2\}$ (10)

where $[E_o]$ and $[I_o]$ are the initial concentration of enzyme and total organophosphate, whereas $\tfrac{1}{2}\,[I_o] = [L_{+,o}] = [L_{,o}]$.

The rate constants k_1 and k_2 were calculated from values of residual enzyme activities determined at various times of inhibition, as follows.

The relationship of residual enzyme concentration and time of inhibition is described as a fourth or fifth order polynomial calculated from the data determined for residual enzyme concentration, derived from the residual enzyme activities, at various times of inhibition:

$[E] = a + b\,t + c\,t^2 + d\,t^3 + e\,t^4\,(+f\,t^5)$ (11)

Next, values of θ are calculated at the various times of inhibition at which residual enzyme activity had been determined, according to

$$\theta = \int_o^t [E]\,dt = \int_o^t (a + b\,t + c\,t^2 + d\,t^3 + e\,t^4)\,dt = a\,t + 1/2\,b\,t^2 + 1/3\,c\,t^3 + 1/4\,d\,t^4 + 1/5\,e\,t^5 \quad (12)$$

in case of a fourth order polynomial.

Finally, equation (10) is fitted to the set of data for [E] and θ obtained in this manner, from which values for k_1 and k_2 are obtained.

BIBLIOGRAPHY OF PUBLICATIONS AND MEETING ABSTRACTS

Publications

VAN DER SCHANS, M.J., VAN DER WIEL, H.J., SPRUIT, W.E.T., TRAP, H.C., VAN DIJK, C., VAN DER LAAKEN, A.L., PLEIJSIER, K., NOORT, D., DE JONG, L.P.A., BENSCHOP, H.P. AND LANGENBERG, J.P. (2002) Effect of pretreatment with human butyrylcholinesterase scavengers on the toxicokinetics and binding of nerve agents in guinea pigs. Proceedings of the 2002 Medical Chemical Bioscience Review, June 3-7, 2002, Hunt Valley (MD), *submitted.*

Presentations

VAN DER SCHANS, M.J., VAN DER WIEL, H.J., SPRUIT, W.E.T., TRAP, H.C., VAN DIJK, C., VAN DER LAAKEN, A.L., PLEIJSIER, K., NOORT, D., DE JONG, L.P.A., BENSCHOP, H.P. AND LANGENBERG, J.P. (2002) Effect of pretreatment with human butyrylcholinesterase scavengers on the toxicokinetics and binding of nerve agents in guinea pigs. Poster presented at the 2002 Medical Chemical Bioscience Review, June 3-7, 2002, Hunt Valley (MD).